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Beyond the lateral flow assay: A review of paper-based microfluidics

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ABSTRACT

The interest in and use of microfluidic paper-based analytical devices (μPADs) has grown exponentially over the last decade. Cellulose, and modified cellulose, has been used for centuries for making chemical measurements but devices made with this material traditionally suffered from either poor detection limits and/or limited ability to provide quantitative measurements. μPADs address these problems by patterning paper to create microfluidic channel networks that can direct flow to different regions of the device without the need for external pumps used in most traditional microfluidic devices. Furthermore, because the devices are made from cellulose or modified cellulose using inexpensive manufacturing methods, the devices can be inexpensive and disposable. The ability to carry out multiplexed analysis without external pumps using inexpensive, disposable devices makes μPADs ideal for point-of-care (POC) analysis. The interest in μPADs has led to a number of excellent comprehensive reviews in the field; in this review, we do not attempt to reference the entire field but instead seek to highlight major developments as well as areas that will be important for future development of this field.

1. Introduction

Rapid diagnosis of a disease early in its development is critical for effective treatment. For the most serious diseases, timely diagnosis is truly a matter of life and death. Unfortunately, if that diagnosis requires the patient or sample to be tested in a centralized laboratory, then the promptness—or even the possibility—of diagnosis is greatly diminished in resource-limited settings [1,2]. For people living in regions that lack necessary technical and economic resources, clinical lab tests may be practically out of the question. Either the expense or the distance involved, or the lack of skilled medical personnel to direct the process will preclude such laboratory testing, at least in a timely manner [3]. For that reason, the development of simple devices to reliably and rapidly perform diagnostic testing outside the laboratory is vital. It is estimated that the availability of reliable point of care (POC) diagnostic devices for just four infectious diseases—bacterial pneumonia, syphilis, malaria, and tuberculosis—could prevent at least 1.2 million deaths worldwide each year [1,3,4].

POC diagnostic devices are not generally intended to replace more sophisticated laboratory tests, but rather provide a means of rapid initial screening in non-laboratory settings. Since simple diagnostic devices can be used at the POC, even if remote, they minimize the delay and expense inherent in the requirement for a testing laboratory. The more costly and centralized laboratory tests may then be used judiciously to confirm screening results and provide more detailed information as needed [1–4].

The World Health Organization (WHO) recognizes the importance of POC devices in diagnosing diseases and has established criteria for evaluating such medical diagnostics. Given the acronym “ASSURED,” the criteria specify that appropriate devices should be “affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end users” [5]. In the same context, WHO also stipulates that “such a device should be operable in resource-limited environs, which include those with unreliable electricity, non-sterile conditions and a lack of trained personnel to perform the duties typically reserved for nurses and health workers” [5].

Although originally developed specifically for human immunodeficiency virus (HIV) testing, these WHO criteria are broadly applicable to any diagnostic devices for resource-limited settings. The need for such devices extends beyond the realm of medical diagnosis to other concerns in food quality [6] and environmental testing [7,8], both of which also impact human health. In each case, the simpler and less expensive the testing device, the better, as long as it is sufficiently accurate and reliable.

To adequately address WHO considerations, analytical tests generally need to be miniaturized and simplified. Therefore, the use of microfluidics—e.g., directed fluid flow, immobilized reagents, and/or sequential mixing in miniature channels—is well-suited to such diagnostic devices. Microfluidic platforms have been made from a wide variety of materials (e.g., silicon, glass, acetate transparencies, polydimethylsiloxane (PDMS), poly(methyl methacrylate) (PMMA), and other polymers), each with particular advantages and disadvantages.

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Because of its availability, low cost, ease of safe handling and disposal, and inherent capillary flow, cellulosic paper has been especially popular [9–11]. Depositing reagents and creating flow channels in paper is relatively simple, given the availability of ink-jet and wax printers and the permeability of paper. Such microfluidic paper-based analytical devices (μPADs) have undergone many innovative developments over the last decade, developments from which one can identify several important common themes and future directions.

There have been numerous reviews of microfluidics and specifically μPADs in recent years [10–17]. While previous reviews have provided an extensive listing of recent publications in the field and thus provide an overview of μPAD developments and applications, this review is more limited in scope. In it, we attempt primarily to address two questions: 1) in what directions is the μPAD field moving in the next decade, and 2) what are the field's most significant strengths, challenges, and potential solutions to these challenges? In other words, this review is focused on identifying several of the most important considerations that arise and will likely be key issues in the future development of viable μPADs for broad use. We recognize that we may, by limiting our scope, inadvertently omit references to some excellent work in the field. Readers are therefore referred to the reviews cited above for a more extensive listing of recent μPAD research.

In looking to the future, it is often helpful to consider how a given field has evolved thus far, so we first provide a brief review of the historical development of paper-based analytical devices. We then turn our attention to three major application areas for μPADs: medical diagnostics, environmental testing, and food quality assessment. A small subset of representative articles from these areas exemplify definitive strengths and challenges in the field moving forward. From these examples, we extract what we see as significant themes common to μPADs that will be important considerations in future developments and improvements in the field.

2. A brief history

Science has had a centuries-long interest in the use of woven cellulose fiber networks for performing chemical measurements. While there were a few instances of this type of application in earlier eras, a major milestone was reached around 1700 when litmus paper began to appear. Litmus paper, a simple piece of cellulose paper impregnated with pH sensitive chromophores, was a significant revolution at the time because it enabled the measurement of pH in places and samples where such measurements had not been previously possible [18]. Inspired by this concept, West developed spot tests for metals in the 1930s and 1940s where the pH sensitive chromophores were replaced with ligands that changed color in the presence of specific metals [19]. While these modified paper strips lacked sensitivity and sometimes selectivity, they provided an important step forward in the development of analytical tests that could be performed in the field. These key steps laid the foundation for the development of the most widely used form of paper device: the lateral flow assay (LFA). LFAs appeared in the 1970s and remain a cornerstone of modern medicine used to detect a wide range of pathogens and biomarkers. They typically utilize labeled antibodies to capture and detect a biomolecule through a colorimetric or fluorescent readout, and are also used in other fields such as environmental monitoring and law enforcement [20]. In LFAs, fluids are transported via capillary action along a nitrocellulose strip that is preloaded with reagents. The chemistry and physical shape of the fibers helps control wetting rate. At the end of the assay time, qualitative and/or semi-quantitative readout is achieved by analyzing a colored band [21].

Arguably the most successful and globally significant point-of-care medical diagnostic device was also being developed in the 1970s, the blood glucose monitor for diabetes patients [22–25]. This device utilizes an immobilized enzyme (glucose oxidase or dehydrogenase) on a disposable test strip containing electrodes. A small drop of blood is added, the enzyme catalyzes oxidation of the glucose, and the meter measures the associated current produced (either directly or via a redox mediator). All the necessary reagents and even the electrodes are included within the disposable strips, yet the strips are quite inexpensive (typically less than $0.10 each). The enzyme reactions are highly selective and sufficiently sensitive for routine, rapid blood glucose monitoring throughout the relevant clinical range. Furthermore, the strips are stable for a year or more at temperatures up to 45 °C. Modern glucose meters are battery-operated, affordable, handheld, simple to use, and small enough to be carried in a small purse or even a pocket. The primary disadvantage of the blood glucose monitor is the requirement for a small drop of blood as the sample; however, continuing development has minimized the volume needed to a few microliters, obtainable via a very small pin prick in the patient's skin. Alternative non-invasive, non-blood sampling techniques are also being commercialized. Blood glucose monitors are used throughout the world by millions of people daily. Thus, in many ways, the blood glucose monitor represents the “gold standard” of POC diagnostic devices.

With these major developments as a backdrop, the first μPAD appeared in 2007 through work from the Whitesides laboratory [26]. What distinguished this research from prior work on LFAs and spot tests was the use of patterning to create flow channels. In traditional microfluidics (with a few notable exceptions), flow is generated through either hydrodynamic pressure or electroosmotic flow. Paper generates flow through capillary pressure induced by wetting of cellulose fibers that form capillaries in the material. Although these forces produce flow in LFAs and spot tests as well, μPADs utilize patterning to create *directed* flow. While seemingly a modest contribution, the ability to direct flow in a manner similar to microfluidics opened many new doors to the field, including the ability to perform complex sample preparation steps as well as multiplexed detection on the device.

From the initial report in 2007, the field rapidly expanded to include many more groups and key developments. New methods for fabricating devices appeared first, with a transition from photolithography (a complicated, expensive approach) to wax printing, screen-printing, and inkjet printing; these, in turn, expanded accessibility of the field to many researchers around the world. New methods for detection also appeared (the initial report used colorimetric detection), with the Henry group pioneering use of electrochemical detection [27], the Whitesides group the use of fluorescence [28], and the Yu group the use of chemiluminescence [29]. Areas of application also expanded from the initial work in point-of-care diagnostics to include efforts in food and environmental testing by the Henry group [30,31]. The μPAD field has grown rapidly to the point where hundreds of articles are published each year describing the latest innovations. Despite this substantial growth, much work remains to move μPADs from academic curiosities to practical solutions for important global problems.

3. Application areas

3.1. Medical diagnostics

Since their introduction in 2007, μPADs have shown tremendous potential as POC diagnostic tools for a wide variety of health issues. Such devices have recently been developed to detect many biomarkers and pathogens, including NO_2^- in saliva, Ebola virus RNA, Salmonella, Hepatitis C antibodies, virus DNA, and glucose [32–37]. While numerous diagnostic μPADs have been reported in the literature, there are pitfalls common to many that make them non-ideal for their intended use at the point-of-care in resource limited settings. These pitfalls include: 1) poor detection limits obtained in μPADs are particularly troublesome for biological analytes which are often relevant at sub-attomolar concentrations [38], 2) common reagents used in biological assays, for example, antibodies and enzymes, degrade easily in resource limited settings where storage conditions such as temperature and humidity are difficult to control [39], and 3) most existing μPADs test for one specific analyte rather than a suite of analytes. These problems will

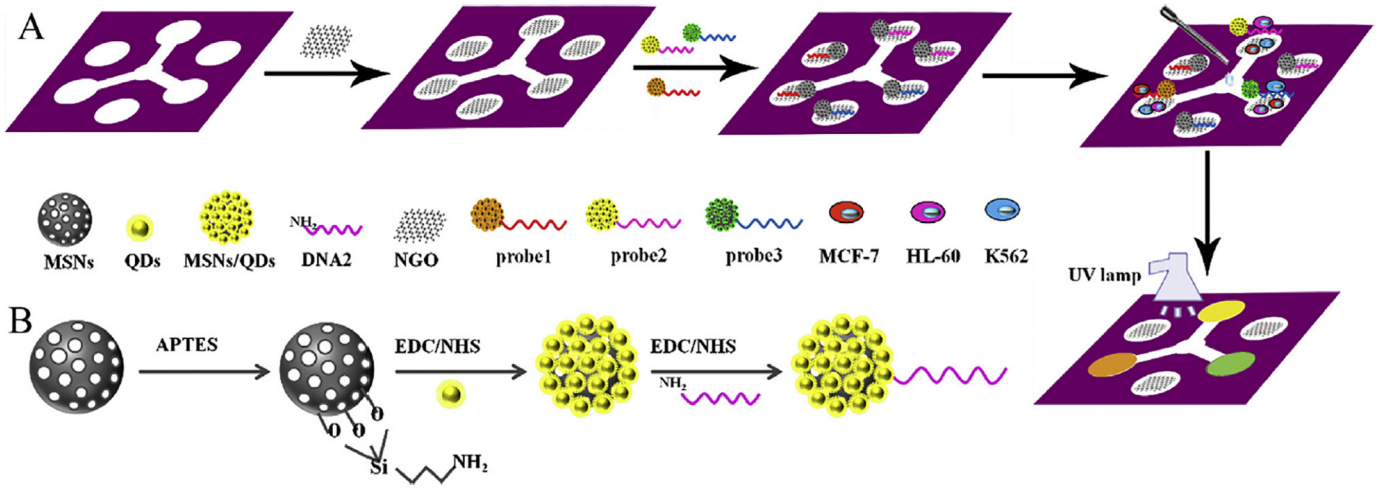

Fig. 1. Schematic representing the fabrication process of the multiplexed μPAD and quantum dot-coated silica beads developed by Liang et al. A.) μPAD consists of three negative control areas that are not connected to the sample inlet and three sample wells each containing a different QD/aptamer pair. B.) Functionalization of silica nanoparticles with QDs and aptamers.

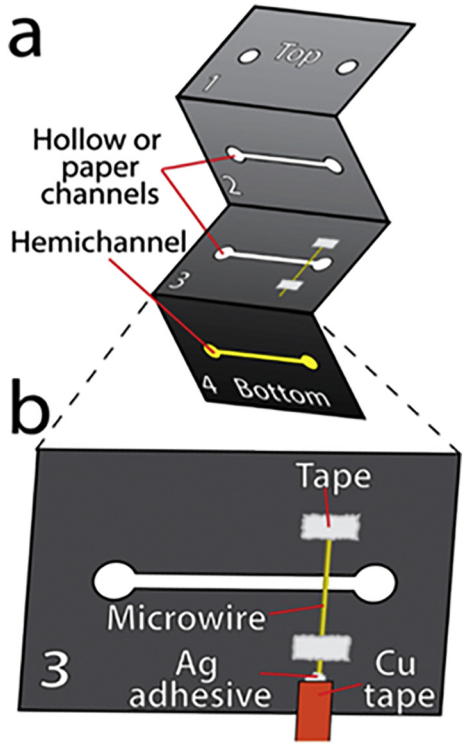

Fig. 2. Schematic representing the fabrication of the hollow channel origami PAD (HC-*o*PAD). A) Layers 1 through 4 of the HC-*o*PAD. B) Incorporation of the microwire electrode into the hollow channel device.

be important to address before μPADs can become commercialized POC diagnostic tools. Therefore, in the coming years we expect and advocate for μPAD research in this area to focus on improving sensitivity and detection limits, developing new techniques for long-term and stable storage of reagents on paper in uncontrolled conditions, and fabricating devices capable of multiplexed testing. We also expect the field to move towards preventative screening in addition to acute diagnostics, as healthcare experts have promoted in recent years [40].

Many groups have addressed the pitfalls listed above by integrating new sensing motifs on μPADs. For example, an aptamer-based fluorescent μPAD capable of multiplexed screening of cancer cells was developed in 2016 (Fig. 1) [41]. This paper device is coated with graphene oxide and quantum dots (QDs) that are functionalized with DNA aptamers. The aptamers target specific lines of cancer cells but also bind to graphene through π-π stacking, effectively quenching the fluorescence of the QDs. When the aptamers bind to their respective cancer cells, the QDs are unquenched and fluoresce. Each cell line is represented by a different color QD, so the color of the fluorescence indicates the cell type detected. The device can be used with the naked eye for qualitative testing but can also be used with a fluorescence detector for sensitive quantitative measurements with low detection limits [41]. Sensitivity was enhanced by coating mesoporous silica nanoparticles with the QDs. The nanoparticles have a large surface area-to-volume ratio, which increased the number of QDs available for binding with cells on the surface of the device, compared with QDs adsorbed on paper alone. This work is an example of μPAD research focused on preventative screening, improving sensitivity and LODs, and multiplexed testing. However, the reagents require refrigerated storage, which is problematic in resource-limited settings.

As shown above, LODs on paper can be improved over traditional colorimetric detection through techniques like fluorescence. Another method often used to lower detection limits is electrochemistry. However, in clinical diagnostics, these sensitive detection methods may still require some form of chemical amplification, such as enzyme or metal ion-based enhancement, to further lower detection limits to clinically relevant levels [42,43]. While such detection limits have been achieved, the chemical amplification procedures are often unattractive in μPADs as they require expensive reagents, multiple steps to be carried out by the end user, and complex device fabrication methods.

In the earliest iterations of electrochemical μPADs, electrodes fabricated directly onto paper suffered from lowered sensitivity, by up to 40%, as a result of the cellulose fibers occupying a significant area of the electrode surface [44]. This problem is associated with both screen/stencil printed carbon electrodes (SPCEs) and sputtered or printed metallic electrodes. Recently, Crooks et al. published several articles on their work with hollow-channel μPADs [42,45,46]. The hollow-channel μPADs, shown in Fig. 2, demonstrated the incorporation of microwire electrodes in μPADs for the first time. Hollow-channel μPADs with microwires provide ePADs with: 1) the capability to perform bulk solution electrochemistry under flow conditions; 2) electrodes which are amenable to surface modification external to the device, allowing for harsher pre-treatments without damaging and/or contaminating the paper substrate; and 3) faster flow rates than those obtained in single layer μPADs, permitting faster analysis times [47,48]. Specifically, important to clinical diagnostics, Crooks et al. demonstrated the

applicability of gold microwire electrodes modified with self-assembled monolayers (SAMs). SAMs are used extensively in electroanalysis of complex biological samples in order to achieve high sensitivity, high selectivity, and low detection limits of the target analyte [47]. In our opinion, this work represents a significant milestone in the current and future development of ePADs capable of reaching the detection limits required for clinical diagnostics without the need for complicated chemical amplification methods.

The Henry group recently incorporated SAMs-modified gold microwire electrodes into a two-layer ePAD with quasi-steady flow providing highly sensitive and selective virus particle detection via electrochemical impedance spectroscopy (EIS) [48]. EIS is an attractive detection method in biosensing as it can detect the binding events that occur on a transducer surface. Surfaces can be modified to impart high selectivity to the binding of the target analyte, which is desirable in complex biological samples. Also, while EIS detection retains the sensitivity associated with electrochemical PADs, it is not necessary for the target analyte to be a redox-active species [49]. For these reasons, this work demonstrates significant progress in point-of-care biomedical diagnostic devices which provide low detection limits while retaining low cost, simple fabrication and operation, and high specificity. A fan shaped 2-layer device was used to generate quasi-steady flow rates for EIS detection [48]. Gold microwire electrodes were modified with SAMs containing bioaffinity reagents specific to the binding of the target analyte through a stepwise bioconjugation process. Each binding event in the process was shown to produce an increase in the measured impedance or resistance to charge transfer of a model redox mediator as shown in Fig. 3. Lower detection limits of the model analyte, strepdavidin particles, were achieved using the flow based PAD as a result of increased mass transfer of species to the electrode surface under flow conditions as opposed to static conditions. The ePAD was then used to detect the West Nile Virus (WNV) with a detection limit of $2.04*10^3$ WNV particles per mL, or about 10 WNV particles per 50 µL of sample. This detection limit is comparable to those achieved with methods such as RT-PCR and ELISA and is in the clinically relevant range of $1.0*10^2$ to $1.0*10^6$ genomic equivalents per mL. The clinically relevant range is dependent on sample type (tissue location and blood/urine) as well as stage of infection. The ePAD developed in this work represents what we believe to be a promising direction in ePAD research to provide fast, simple, robust, inexpensive, specific and highly sensitive point-of-care biomedical diagnostics. As research on new applications for µPADs continues to progress, we expect and advocate for the integration of sensing techniques like electrochemistry and fluorescence to improve detection limits and increase practicality of paper-based diagnostics in clinical settings.

3.2. Environmental testing

In environmental analysis, it is important to consistently monitor and evaluate the concentration of analytes in natural settings such as water, soil, and the atmosphere. These analyses range from simple (e.g., pH measurement), to more complex (e.g., multiplexed detection of heavy metals). The portability of µPADs makes them ideal for in-field testing, especially within the environmental sector. Analytes can be analyzed on site, eliminating the need for large sample volumes to be transported back to a centralized laboratory. However, there are still challenges associated with the optimization of these devices, including the need to improve sensitivity, specificity, reagent stability, and on-site data analysis. The devices need to detect analytes at levels at or below those set by regulatory agencies such as the U.S. Environmental Protection Agency (EPA) and the World Health Organization (WHO). This is especially critical for compounds that have negative effects on living organisms [50,51]. The tests must also be highly selective and unaffected by complex sample matrices [52,53]. Multiplexed testing has been achieved on µPADs, but depending on the analytes, pretreatment may be required which increases cost and time, and decreases ease of use [54,55]. Reagent stability is also critical, since the devices may need to be transported and used under a wide range of temperature, humidity, and sunlight conditions [52,55,56]. Finally, there has been significant progress in data processing, moving from large, expensive, lab-based instruments to smartphones and image analysis software. However, there are still many variables that need to be controlled to ensure reproducible data processing [51,52,55].

In 2014, an Android smartphone application was developed to process images of a paper-based device for measuring nitrite concentration and pH of water samples (Fig. 4). This work demonstrates the ability to detect multiple analytes on one device and to obtain immediate results in the field. Nitrite is an indicator of bacterial contamination, while pH is one of the main indicators for the health of general plant and aquatic life [51]. In this device, Griess reagents immobilized on the paper reacted with nitrite to produce a color change from colorless to pink, allowing quantification. The pH was measured

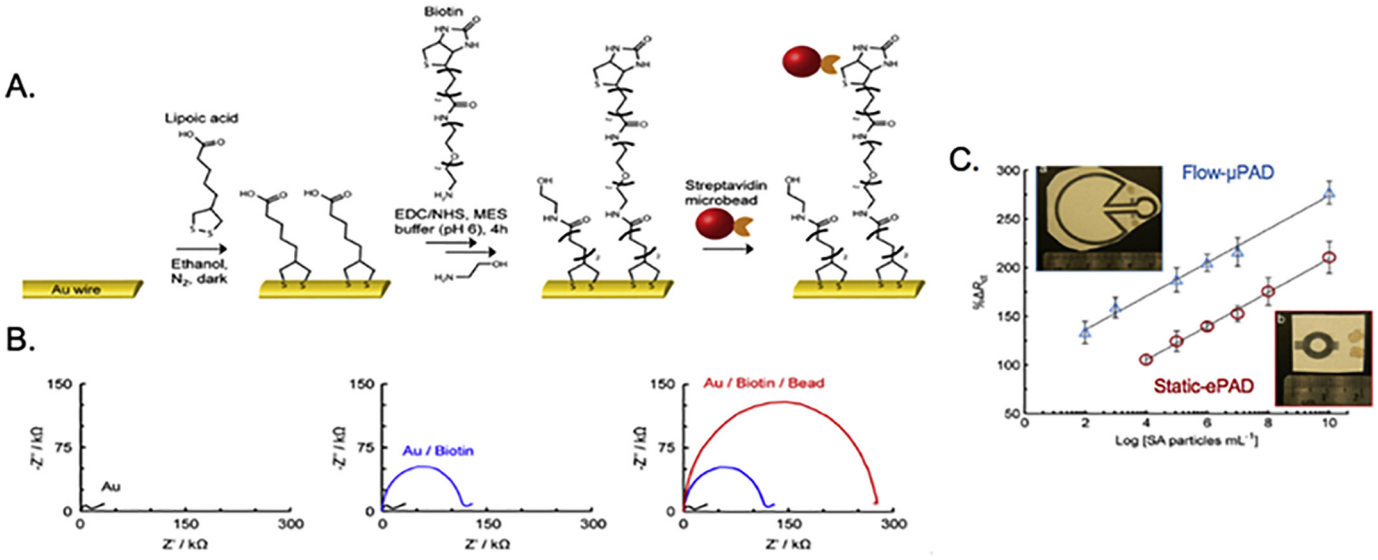

Fig. 3. A) Scheme showing the reaction steps for modification of Au microwires with SAMs which are chemically modified to selectively capture streptavidin microbeads. B) EIS Nyquist plots at different steps of the modification/capture process. C) Calibration curve for the detection of streptavidin microbeads under flow and static conditions.

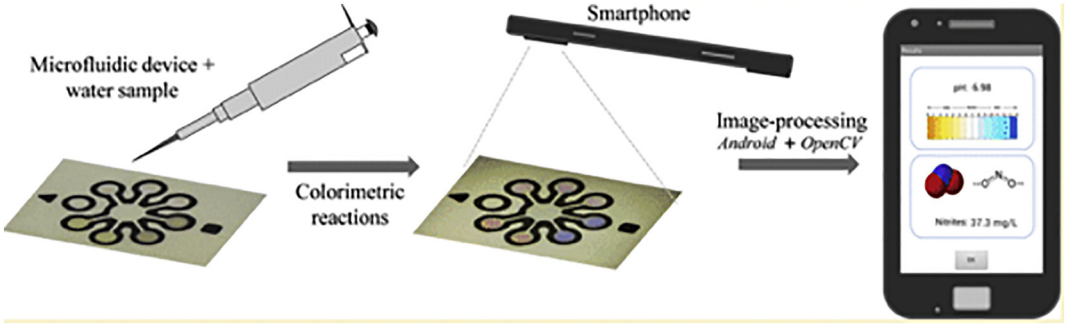

Fig. 4. Scheme showing use of the paper-based device to determine pH and nitrite concentration with analysis by smartphone [51].

by a color change in two pH indicators stored on the paper that cover the pH range of interest: phenol red and chlorophenol red. The device only requires that one drop of water sample be placed in the sample inlet and then the capillary action of the paper wicks the sample into the sensing zones. After 15 min, the analytes have reacted with the stored reagents, dried, and are ready for analysis.

The Android application ensures that camera placement is consistent for each sample by aligning marks on the device and uses the camera flash for consistency in lighting. The change in the hue and saturation coordinates of the hue-saturation-value (HSV) color space combined with a custom algorithm give a final readout. The μPAD and Android application obtained comparable results to a standard potentiometric method for pH determination; however, it was less accurate for nitrite analysis. This was due to non-uniform absorption of the sample on the sensing area and could be improved in further iterations of the device. This work demonstrates successful, reproducible, multiplexed detection. However, the device's LOD for nitrite (0.52 mg/L) needs to be improved. While it falls within WHO's acceptable concentration of under 3 mg/L, the European Commission limit is 0.5 mg/L. A lower detection limit would be preferred to confidently quantify the nitrite concentration in water and evaluate its potability. Despite these limitations, the work demonstrates the development of a smartphone application that provides consistent, objective analysis of colorimetric data in the field.

Heavy metals are another significant group of environmental contaminants. A μPAD was developed for metal quantification in aerosolized particulate matter (PM) filter samples, using both colorimetric and electrochemical detection methods [53]. The multiplex device combined two types of detection to allow detection of six heavy metals. Separated detection layers in the device improved sensitivity and selectivity. Fabrication of the multilayer sensor included wax printing the device pattern onto paper and screen printing electrodes onto a polystyrene film (Fig. 5). The dual detection system allowed for the determination of Fe^{3+}, Ni^{2+}, Cr^{6+}, and Cu^{2+} using a colorimetric method and Pb^{2+} and Cd^{2+} using square-wave anodic stripping voltammetry. For colorimetric detection, each detection zone is preloaded during fabrication with the appropriate reagents to produce a color change with each metal individually. The colorimetric reactions of Ni^{2+} with dimethylglyoxime (DMG), Fe^{3+} with phenanthroline, Cr^{6+} with 1,5 diphenylcarbazide (1,5 DPC), and Cu^{2+} with a mixture of bathocuproine and polyethylene glycol (PEG), have been reported individually before, but were combined here for multiplexed detection. The lowest detectable mass reported for Fe^{3+}, Ni^{2+} and Cu^{2+} were 0.75 μg and for Cr^{6+}, 0.12 μg. To detect Cd^{2+} and Pb^{2+}, anodic stripping voltammetry (ASV) was used with bismuth and ferricyanide additives. Bismuth was used to improve ASV performance, as it allowed for the stripping measurement to be done without deoxygenation by forming metal-Bi alloys at the electrode surface. Ferricyanide was added to reduce interference from Cu^{2+}. 0.25 ng was reported as the detection limit for both Cd and Pb. An interference study for Cd and Pb was done to take into account many other metals existing in particulate

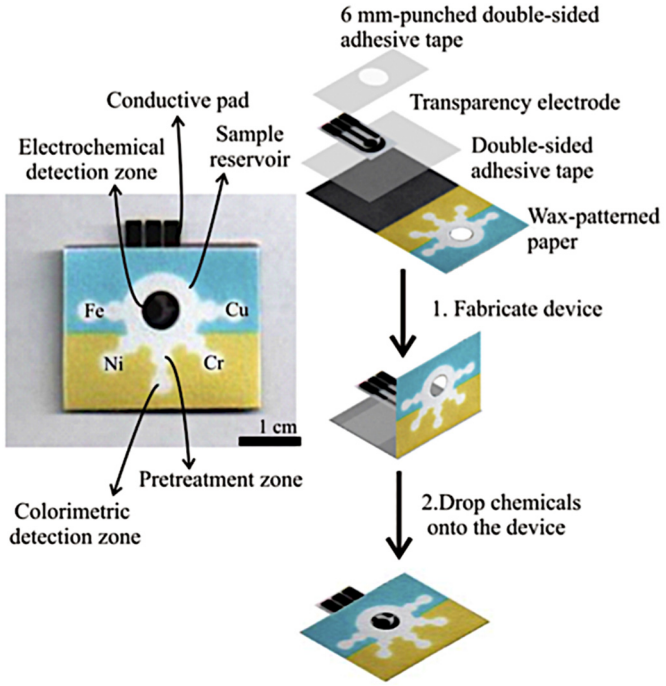

Fig. 5. (Left) Photograph of a μPAD with the different regions labeled. (Right) Schematic of the fabrication procedure for the μPAD [53].

matter (PM) that could potentially interfere with ASV. The study concluded that Mn^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Ba^{2+} and V^{3+} did not affect Pb and Cd detection; however, Ni^{2+}, Co^{2+}, Cu^{2+} and Cr^{6+} caused interference, decreasing sensitivity of the device. To minimize these interferences, the authors proposed dilution of aerosol samples (decreasing filter sample amount and maintaining extraction volume), and complexation of Cu^{2+} with ferricyanide. The authors showed use of the dual sensor by analyzing filter samples for known amounts of Cd, Cr, and Pb in the presence of various metal interferences [53]. The device was successfully able to measure the amount of each metal in laboratory-generated PM filter samples within an uncertainty of 0.01 μg and 0.01 ng for colorimetric and electrochemical detection, respectively.

Fig. 5. (Left) Photograph of a μPAD with the different regions labeled. (Right) Schematic of the fabrication procedure for the μPAD [53].

Ammonia is another prevalent atmospheric component with ambient concentration in the range of low ppb to sub-ppb levels. In 2015, a μPAD was developed to measure ammonia in wastewater samples by employing gas-diffusion separation on paper [52]. The gas-diffusion μPAD was fabricated by adding sodium hydroxide in the circular hydrophilic sample zone and adding the acid-base indicator to the detection zones (Fig. 6) [52]. The sample required no additional reagents

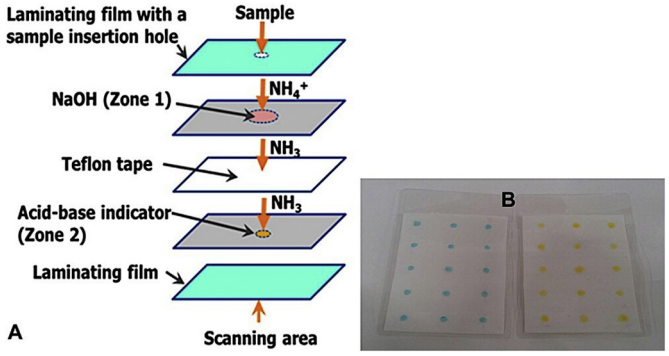

Fig. 6. (A) schematic of fabrication diagram of the gas-diffuse μPAD. The diameter of zones 1 and 2 are 7 and 3 mm, respectively. (B) Photograph of the detection zone side of a bromothymol blue μPAD (left) and a 3-nitrophenol μPAD (right) [52].

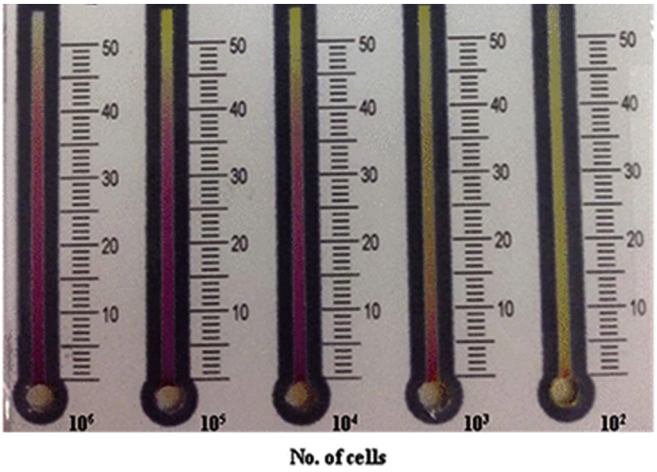

Fig. 7. Distance-based "chemometer" for *salmonella* detection [35].

or pretreatment. Furthermore, analysis was fast (2–6 min), comparably sensitive to commercially available sensors (working range of 10–100 mg/L), and highly selective when used to test soil and waste water. The cost per analysis with the proposed μPAD was determined to be less than half of that for current commercial sensors. The interference of methylamine in wastewater samples was determined to be negligible. The stability of the sensor depended on the pH indicator used. When 3-nitrophenol blue was stored on the device, the reagent was stable for up to 8 days at room temperature while bromothymol blue lasted up to 3 months under the same conditions. Despite bromothymol blue providing optimal stability at room temperature, it resulted in a higher detection limit of ammonia (106 μM) than with 3-nitrophenol blue (47 μM). Overall, compounds like nitrite, heavy metals, and ammonia are just a few examples of analytes of interest that are targeted to monitor in water, soil and atmospheric samples. Paper-based analytical devices have shown major potential, specifically within the environmental sector. With further research, limits of detection, reagent stability, and specificity can be improved, making the devices more useful.

3.3. Food quality

As the worldwide population continues to grow, so does the necessity to increase food production. However, as food production increases, efficient food quality monitoring methods to combat foodborne illnesses become more important. Recently, μPADs have been reported that use fluorescence, colorimetry and/or electrochemistry to detect analytes related to food safety. These articles assess various analytes such as amine vapors from fish, mycotoxins in cereals, and bacteria [35,57–65]. However, bacteria related assays are the most common food quality applications. Bacteria such as *Escherichia coli* (*E. coli*) and *Salmonella typhimurium* (*Salmonella*) are commonly found in food and water, causing over a million foodborne illnesses per year in the United States alone [35]. Adkins et al. used two types of μPADs to indirectly detect *E.coli* and *Enterococcus spp.* [57]. To accomplish this, enzymes (β-galactosidase, β-glucuronidase, and β-glucosidase) released by the bacteria were reacted with substrates that produced *p*-nitrophenol (PNP), *o*-nitrophenol (ONP) and *p*-aminophenol (PAP). The PNP, ONP, and PAP were detected colorimetrically with a spot test and electrochemically with an ePAD. While maintaining a small, inexpensive, and easy to use system, good in-laboratory calibration curves were obtained with these two μPADs. Despite obtaining low LODs (2.3×10^2 CFU/mL of *E. coli*) for real samples, the LODs between colorimetric and electrochemical detection were similar [57]. This was unexpected since electrochemical detection schemes generally provide lower LODs than colorimetric ones. A major disadvantage of this system was the necessity to enrich real samples by growing the bacteria to generate concentrations detectable via the described methods. Unfortunately, the 4–24 h pre-enrichment process required seven steps prior to quantification, increasing both the analysis time and complexity of the assay.

Srisa-Art et al. reported a colorimetric spot test and distance-based μPAD for quantifying *salmonella* [35]. The spot test here is similar to the work discussed above. In addition to the spot test, a "chemometer" device was developed that measures the length of a color band to quantify an analyte (Fig. 7). This read-by-eye format is a simple detection method which does not require external instruments such as a camera or potentiostat. This article also describes an immunomagnetic separation (IMS) technique that allows for specific binding and separation of an analyte from complex matrices. The method does not require pre-enrichment steps, uses room temperature incubation, and real samples can be analyzed in 90 min. Despite more rapid quantification, this method still requires over thirteen steps to carry out the immunomagnetic separation. In addition, the analysis of real samples increases the LOD from 10^2 to 10^3 CFU·mL^{-1} and the end-point for the color band in the chemometer requires subjective determination [35].

Park et al. used delayed flow and channel partition formation on a nitrocellulose (NC) membrane to achieve a simple, single-step device for on-site detection of *Salmonella*. During fabrication, the paper is compressed to reduce its pore size and thus the fluid flow rate. The change in pore size can be modified and controlled by the amount of pressure applied. Two types of antibody-conjugated gold nanoparticles (AuNPs) are used for each pathogen being detected, and three gold enhancer components (i,ii,iii) are preloaded and dried on the pressed NC membrane. The gold enhancers are used to amplify the colorimetric signals. This one-step operation device uses a compressed dipstick with dehydrated reagents to carry out a multistep reaction scheme. The device is dipped into a sample solution and the analyte reacts with the antibody conjugated gold nanoparticles. The immunocomplexes are then captured by antibodies at the test line for colorimetric signals (Fig. 8). The colorimetric signals are analyzed with ImageJ software and the concentration is determined for each analyte. As a proof of concept, the group detected *E. coli* and *Salmonella*. Detection limits of 10^6 CFU·mL^{-1} for *Salmonella* and 10^5 CFU·mL^{-1} for *E. coli* were obtained. This is comparable to current USDA standards of approximately 10^5 CFU·mL^{-1} for these bacteria, but detection limits of 10^2 CFU·mL^{-1} or lower are often expected for other bacterial species. Further optimization of areas such as concentration of capture antibodies, rehydration conditions of dried reagents, and device dimensions could potentially improve detection limits. Despite showing simplicity in operation, some challenges faced were a poor limit of detection with a high standard deviation in signal intensity. This work demonstrates a potential method for a simple, one-step, and user-friendly assay for detection of *Salmonella*. However, this device needs to be more precise

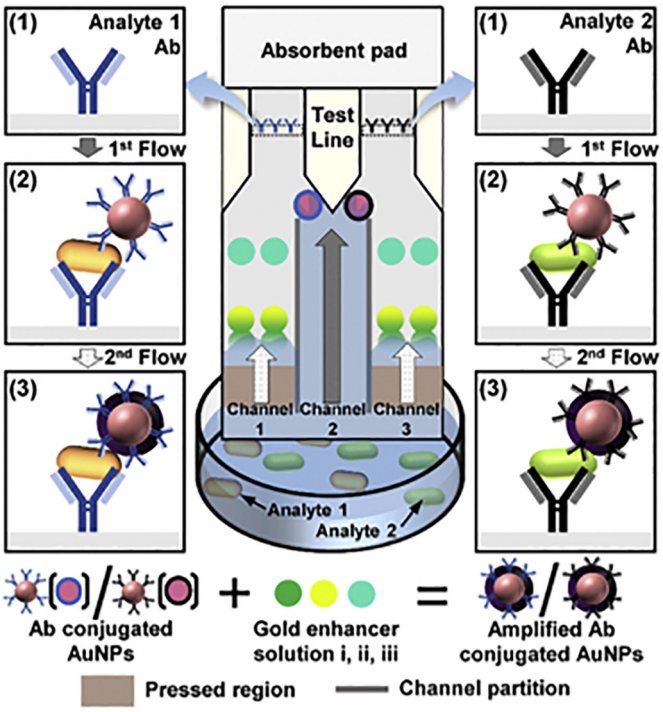

Fig. 8. Step by step operation of pressed paper dipstick device [65].

and sensitive for it to be applicable in the field [65]. Simple one-step asssays will continue to be desirable for paper based devices because of their ease of use in non-laboratory settings. The articles discussed above are just a few of the many published in which researcers are attempting to develop more efficient means of food mointoring in resource-limited areas to prevent foodborne illness.

3.4. Current industrial applications

Although many traditional paper-based devices such as dipsticks, pH paper, and LFAs have been successfully commercialized, there are few current examples of μPADs on the market. Many of the reasons for the absence of commercialized μPADs have been discussed in this review; however, the lack of funding beyond the initial assay development stage is also a common limiting factor. Of the companies that have developed commercial μPADs, many are working on medical diagnostic assays, which are projected to have a market value of over $8 billion by 2022 [66]. Diagnostics for All (DFA) is a company that has produced a wide array of paper-based diagnostics that monitor liver function, detect HIV, and even help determine when a cow goes into heat. Haemokinesis and INSiGHT are two other companies that have developed paper-based tests for blood typing and multiplexed antibody detection, respectively [67]. Another way to bridge the lab-to-field gap is through collaboration between well-established corporations and academic institutions. Intellectual Ventures is a company that works with academic labs for product development and with commercial partners to manufacture and deploy products. Although Intellectual Ventures works with a wide variety of industries, it is currently helping create paper-based tuberculosis and malaria tests. Other companies are designing μPADs for environmental testing. Access Sensors Technologies has created a set of μPADs to measure heavy metal contamination in water. After depositing a small water sample on a test card, a smart-phone application measures the radial area of color change and reports concentration (Fig. 9) [68].

4. Common themes – strengths, challenges, and future directions

While there are many different formats, reaction types, and detection strategies for paper-based microfluidic devices, as exemplified in the application areas discussed above, several common themes emerge. Characteristics such as simplicity of use, low cost, and portability bode well for the utility of μPADs outside of the laboratory setting in resource-limited locations. Indeed, these properties have been largely responsible for the considerable recent interest and surge in research in the μPAD field. As the field continues to develop and grow, it is important to remember that simplicity, portability, and affordability are fundamental expectations of such devices for most applications. WHO ASSURED criteria, described in the Introduction, provide an important perspective that should be kept in mind when considering the practical implementation of μPADs. Compromising one or more of these criteria to solve other challenges presented by μPADs is often less than ideal. However, there are several challenges, summarized below, that also emerge as common themes, especially when developing devices for quantitative analyses. For qualitative applications or rapid initial screenings, some of these challenges are less critical or even irrelevant. For example, excellent precision and sensitivity are not necessary in a device that serves as an initial “yes or no” screening test for a high-concentration diagnostic marker. In other words, the analytical specifications required for a μPAD will often be application-dependent.

4.1. Sensitivity and reproducibility

For semi-quantitative or quantitative analysis, poor sensitivity (yielding high limits of detection) and lack of reproducibility—at least under uncontrolled “real-world” conditions—are among the most significant and commonly encountered issues with μPADs [13,69]. Visual colorimetric detection often suffers from these two problems, so the development of more sensitive and precise detection methods is appropriate and likely to continue to be an important area of research. Electrochemical and luminescence detection methods offer the potential for enhanced sensitivity; however, they also tend to add cost and complexity [70–72]. Ideally, the increased complexity can be incorporated into the design and production of the devices without significantly increasing their cost or diminishing their “user-friendly” characteristics. The use of the μPAD itself should be straightforward and not require multiple steps or manipulations that invite user error and variability. Portable blood glucose monitors for diabetes assessment, developed decades ago, exemplify the possibility of producing a relatively complex electronic device that is easy to use and accessible to many patients across the globe. Alternatively, commonly available technologies such as smartphones or scanners can make colorimetric detection more reproducible, albeit also with some loss of simplicity compared to visual assessment.

One fundamental source of imprecision within many μPADs, is the inherently constrained and variable dissolution of dry reagents stored on the paper matrix [13,69,73]. Of course, in some cases (e.g., an immobilized enzyme), the reagent is intended to remain in place on the paper, and in those cases any loss through dissolution is undesired. In other cases, rapid and uniform dissolution is required. The rate at which a reagent dissolves, the completeness of that dissolution, and the homogeneity of the resulting solution are dependent on several factors, many of which are difficult to control in non-laboratory settings. For example, the inherent solubility, particle size and morphology of the reagent; the volume, temperature, flow rate and turbulence, viscosity, pH, and ionic strength of the solvent; and the porosity, purity, thickness, and uniformity of the paper can all impact the dissolution [73,74]. While some of these properties can be controlled at the time of manufacture, several will be subject to change during long-term storage and use of the μPAD, particularly in resource-limited settings.

In addition to dissolution or rehydration issues, the mixing of two or more moving fluids within a paper matrix needs to be considered for

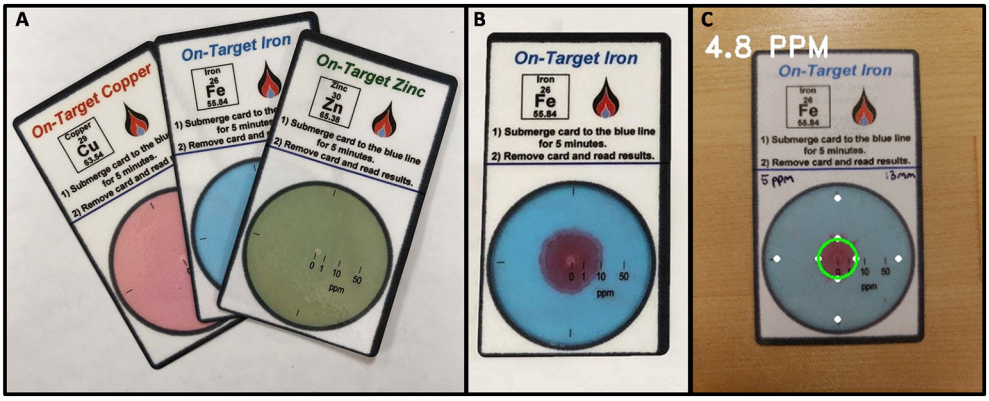

Fig. 9. (A) Current commercial test cards available for purchase include Fe, Cu, and Zn. Tests for Cd, Mg, Cr, Pb, Al, and Phosphate are under development. (B) As water wicks radially outward from a hole in the center of the laminated test card, the diameter of color change indicates the concentration of the specified metal. There are tick marks on the card for naked-eye qualitative measurements. (C) For more quantitative results a smartphone app will measure the total area of color change and display a concentration on screen.

some devices. One cannot presume that two miscible fluids combined within a paper matrix will inherently and rapidly mix to homogeneity. Such mixing tends to be slower and less uniform than mixing in an open container [75]. Thus, improving the speed and homogeneity of dissolution and fluid mixing within paper or paper-like materials is and will continue to be a key area of research. Promising work in this area has been reported [73–75], demonstrating that fluid flow control and uniformity in paper can be enhanced by relatively simple means that do not require instrumentation or that use self-contained battery-powered circuitry. Other non-ideal properties of paper, such as adsorptive retention of analyte, contamination, or reactivity under extreme conditions, while not typically primary concerns, will continue to be important considerations, as well. The use of non-aqueous solvents in μPADs also remains an area of continued development [76], since some of the most widely-used channel fabrication methods on paper (e.g., wax printing) are largely limited to aqueous solutions.

4.2. Specificity and multiplexing

Specificity to prevent false positives is also an important consideration, as complex samples from a wide variety of environments need to be tested for specific analytes without interference from matrix components. However, the sacrifice of device and assay simplicity for specificity is ill advised. False positives are less detrimental than false negatives in diagnostic efforts as μPADs will typically serve as an initial screening before more robust confirmatory tests, which will catch false positives. False negatives, on the other hand, can have severe consequences.

Another area for continued development is the capability of a single device to test for multiple analytes (“multiplexing”). It is often the case that an accurate diagnosis of a disease or confirmation of the presence of a particular pathogen or pollutant requires the measurement of multiple substances or parameters. Ideally, such a test could be reliably performed with a single device that simultaneously tests for several key markers. Not only would such multiplexing improve efficiency, it would also enhance cost-effectiveness in many cases. Device operation, however, should remain simple and the multiplexing be built into the device without requiring the operator to perform much, if any, additional manipulation [1,13].

4.3. Stability

Another challenge that needs to be addressed for μPADs to become commercially viable for use in resource-limited settings is storage stability. Many locations that could benefit most from inexpensive and portable microfluidic devices do not have temperature or humidity control. Thus, it is important to develop μPADs that are stable for long periods of time even when stored at elevated temperatures and humidity (e.g., tropical environments). Package engineering may be part of that solution, along with thoughtful consideration of reagents. Additional challenges exist when testing in different environments, as test results often depend on temperature, humidity, and lighting. Therefore, standardization of testing conditions in resource limited settings must be considered when developing μPAD assays [1,77].

4.4. Data interpretation & tracking

Interpretation of the test results should be as straightforward and objective as possible. Subjective visual comparisons of color may be suitable for qualitative evaluation of the presence or absence of particular analytes, but robust quantitative measurements require objective data. The use of accessible technologies (e.g., smartphones) can make the assessment of color, darkness, or intensity more uniform than visual comparisons [17,71]. Also, measurements of distances traveled or numbers of features transformed by a reaction on a paper device also have the potential to reduce person-to-person variability [11].

Another challenge with point-of-care testing in general (not only μPADs) relates to the documentation, storage, and tracking (or “charting”) of test information [77]. Such information may include not only test results, but also experimental conditions, testing protocol, and patient identification and health indicators at the time of the test. The use of portable diagnostic devices outside of a laboratory setting enhances accessibility and timeliness, but typically lacks the quality assurance practices present in a clinical laboratory. While not directly part of the device fabrication process, such charting considerations cannot be ignored if devices are to be used in patient care. The availability of smartphones or other portable digital storage and transmission devices provides one avenue for addressing these concerns, so it is beneficial if μPADs interface smoothly with such technologies. It is also important for commercial viability that μPADs include testing protocols that are well documented and readily standardized.

5. Conclusion

In summary, μPADs show a great deal of promise for meeting critical demands for rapid and simple analytical testing in remote, resource-limited settings. These devices provide a platform for a wide variety of chemical and biochemical reactions and detection schemes that can be employed to assess health status, environmental concerns, and food quality issues. Significant progress has been made in the last few decades with respect to the number of applications and modes of analysis. However, more research is needed to address several challenges that are commonly encountered. These challenges include poor reproducibility, high limits of detection, inadequate specificity, poor long-term storage stability, the need for multiplexing, and subjective data interpretation. Most μPADs successfully address the majority of these challenges; however, it is a rare μPAD that overcomes them all while still meeting WHO ASSURED criteria. Of course, in many cases,

some of the parameters are less important than others to successfully address the specific need at hand. The fact that many research groups worldwide—both academic and commercial—have joined in the task and are rapidly gaining insight into novel ways to address these issues bodes well for the development of even more robust and field-ready μPADs in the future. Such development and, ultimately, commercialization will benefit not just the scientific community and developed nations, but millions of people living in resource-limited regions throughout the world.

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