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# Review of paper-based microfluidic analytical devices for in-field testing of pathogens

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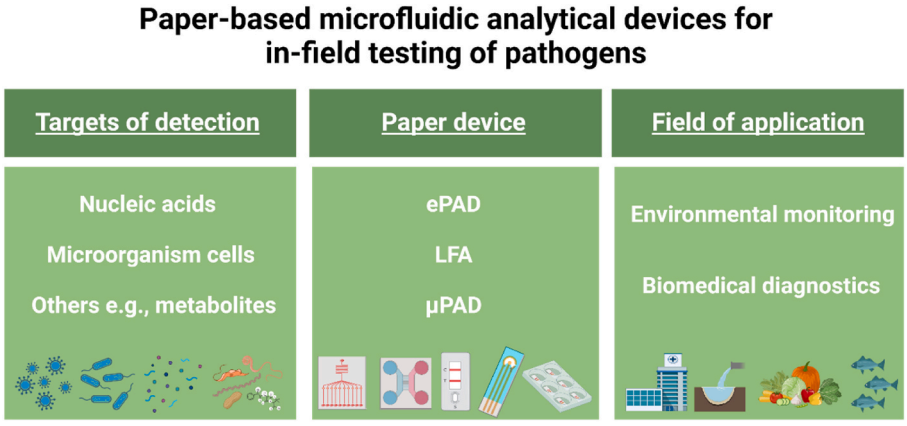
## HIGHLIGHTS

- The state-of-the-art on μPAD is critically reviewed.
- μPAD enables onsite detections of biochemical markers from molecules to organisms.
- Insights is provided highlighting on multidisciplinary aspects.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

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## ABSTRACT

Pathogens cause various infectious diseases and high morbidity and mortality which is a global public health threat. The highly sensitive and specific detection is of significant importance for the effective treatment and intervention to minimise the impact. However, conventional detection methods including culture and molecular method gravely depend on expensive equipment and well-trained skilled personnel, limiting in the laboratory. It remains challenging to adapt in resource-limiting areas, e.g., low and middle-income countries (LMICs). To this end, low-cost, rapid, and sensitive detection tools with the capability of field testing e.g., a portable device for identification and quantification of pathogens, has attracted increasing attentions. Recently, paper-based microfluidic analytical devices (μPADs) have shown a promising tool for rapid and on-site diagnosis, providing a cost-effective and sensitive analytical approach for pathogens detection. The fast turn-round data collection may also contribute to better understanding of the risks and insights on mitigation method. In this paper, critical developments of μPADs for in-field detection of pathogens both for clinical diagnostics and environmental surveillance are reviewed. The future development, and challenges of μPADs for rapid and onsite

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detection of pathogens are discussed, including using the cross-disciplinary development with, emerging techniques such as deep learning and Internet of Things (IoT).

## 1. Introduction

Pathogens posed a significant burden on global public health. For example, bacterial infections caused 7.7 million deaths in 2019, which accounted for 13.6% of global deaths [1]. In addition to bacteria, common pathogens include virus, fungi, protozoa, and helminths, and studies [2–5] have shown their detrimental effect on global morbidity and mortality. Conventional detection methods of pathogens such as observation under microscopes [6] and microbial culturing [7] are both time- and labour-intensive with poor sensitivity. Advancements in modern biological technologies, for example, immunodiagnostics [8–10] e.g., enzyme-linked immunosorbent assay (ELISA) and nucleic acid amplification tests (NAATs) e.g., polymerase chain reaction (PCR) [11], enable faster detection with much less complicated process and better analytical sensitivity, but they are still largely dependent on laboratory setups and expensive equipment, limiting their application in the field where samples are collected. There are also commercial kits such as Colilert are available for monitoring pathogens [12]. They indeed simplify operational procedure, as well as save time and labour, but most of them still require laboratory setup to perform.

Microfluidic devices, first developed in 1979 [13], has contributed to addressing the in-field detection issue. Microfluidics is featured by the integration of multiple functions (sample transfer, mixing, separation, and signal output) in one central unit on the scale of micrometres—the miniaturisation benefits from smaller sample volumes, lower material consumption, and reduced costs and turnaround time. Furthermore, the integration leads to increased automation, and this potential of 'lab-on-a-chip' enables its use to point-of-care (POC)/point-of-use (POU) diagnostics in limited-resource settings. Originating from conventional droplet pattern, microfluidic devices have advanced to digital or paper-microfluidics ones, to meet higher sensitivity, selectivity, portability, and sample throughput. In particular, the μPAD has played an important role in developing biosensors device at low-resource settings (e.g., LMICs) since its first proposal by Whitesides group in 2007 [14].

Paper, as the substrate of biosensors, possesses various advantages [15–17]. It is ubiquitous, and therefore it is cost-effective, suitable for mass production. The lightweight enables its easy transportation and storage. Besides, its cellulose nature gives rise to its intrinsic capillary action, eliminating the need for additional energy source and results in it being compatible with various biomolecules e.g., aptamers and antibodies. Therefore, the paper substrate could be modified easily. Furthermore, the used paper-based sensors could be readily disposed without bio-hazard contamination risks via combustion. Finally, the ability of μPAD incorporating with various detection methods, e.g., optical, magnetic, distance and temperature -based readout, enables to detect a wide range of targets, including microorganisms, nucleic acids, and other biomarkers such as antigens, metabolites and toxins. Followed by the publication of Whitesides group [18], the area of μPAD has flourished with multiple subsequent developments. Dungchai, Chailapakul and Henry published the first electrochemical μPAD for detecting glucose, lactate and uric acid [19]. μPAD can also replace the most popular paper lateral flow assay for detection of hCG [20]. μPAD provides a promising platform to accommodate various bioassays for target recognition and detection. It has not only be employed in clinical settings, but also extends to other sectors including food safety, environmental monitoring, and drug development (see Fig. 1).

In this article, we presented the progress and recent development of μPAD for in field pathogen detection. Firstly, we briefly introduce μPAD, including its substrate, features, fabrication techniques and classifications. Then, we will discuss the progress and development of pathogen detection, including both conventional and emerging onsite approaches. Thirdly, we review the application of μPAD for onsite pathogen detection with various targets, ranging from biomarkers of microorganism-level to nucleic-acid level, and as well as chemicals such as metabolites, and toxins.

## 2. Paper-based microfluidic analytical devices (μPADs) for in-field testing

In the past decades, μPAD has experienced thriving developments

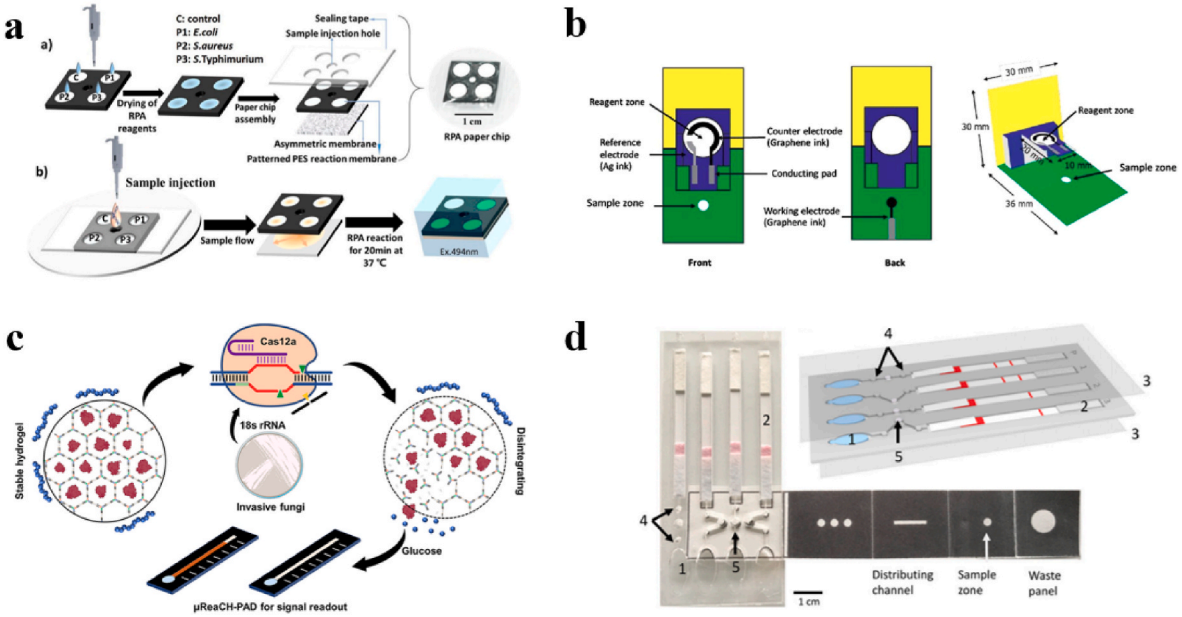


**Fig. 1.** *Examples of μPADs for Escherichia coli, Staphylococcus aureus, and Salmonella typhimurium (bacteria) (a), Hepatitis B (virus) (b), Candida or Aspergillus (fungi) (c), and malaria (parasite) (d) detection. Reprinted from* Refs. [21–23] (Huang et al., 2021).

since its first report by Whitesides group in 2007 [14], as paper was found to bear various merits including affordability, lightweight and popularity, which all contribute to the feasibility of mass production and even potential commercialisation. Besides, its porosity enables ready modification with various chemical and biological reagents. And its capillary effect eliminates the need for external power source, simplifying the device construction. Not to mention the white background colour can also act as a strong contrast in the context of colorimetric detection [15–17]. In general, a μPAD is composed of two parts where hydrophilic channels are surrounded by hydrophobic barriers. As for the choice of the substrate, both unmodified (e.g., filter paper and chromatography paper) and functionalised paper (e.g., graphene and graphite paper) and paper-like cellulose structured material such as nitrocellulose (NC) membrane and mixed cellulose ester (MCE), can be employed as the hydrophilic substrate [15,24,25]. Since the manipulation on fluids and reaction progress are mainly achieved by the design of the barriers, it is of critical importance to understand different fabrication techniques. Table 1 summarises μPADs with different substrate and fabrication methods. It should be noted that most fabrication techniques require extra equipment (e.g., a printer) or steps (e.g., heating) to realise. Photolithography was the first to be employed to pattern μPAD in 2007 [18], where epoxy-based negative photoresist (SU-8) was embedded to create discrete channels. Other fabrication approaches include wax printing, wax jetting, screen printing, and inkjet printing. Featured by its cost-effectiveness, ease of operation, biodegradable nature, and high-resolution patterns, wax printing is regarded as the most utilised method, and various studies are based on it [26]. It should be noted that the wax printer originally employed by the Whitesides group [27], Xerox Phaser 8560 N color printer, is no longer sold. But there are some used ones circulating on the market and recent publications [28,29] still use this specific printer for fabrication. Other types of wax printers have been employed as well e.g., ColorQube8570 [28] or ColorQube8580 [30], with no clear difference in fabrication process and test results.

μPAD can be classified based on its detection approaches, i.e., optical [22,42–45] and electrochemical [19,46], where optical detections can be further divided into colorimetric, fluorescent, and chemiluminescent. With recent developments in technologies such as nanotechnology and CRISPR/Cas system, more potentials on improving analytical performances (i.e., sensitivity and selectivity) and feasibility on in-field detection of μPAD are explored based on their collaboration and integration. Suea-Ngam et al. [47] reported a rapid, sensitive, and quantitative colorimetric silver nanoplates (AgNPls)-based μPAD for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA). A qualitative result by naked eyes while quantitative analysis by a smartphone camera could be realised, with detection limits down to a single copy of related genes in just 30 min, and a linear response from 1 to $10^4$ copies ($R^2 = 0.994$).

The development of CRISPR/Cas technology and its integration on biosensor significantly enhanced the performance while reduced the cost. Tang et al. [48] reported a CLIPON (CRISPR and Large DNA assembly Induced Pregnancy strips for signal-ON detection) system which modifies a commercial pregnancy test strip (PTS) for the detection of nucleic acids and small molecules. They demonstrated the feasibility with quantitative detection of HPV genomic DNA, SARS-CoV-2 genomic RNA and adenosine under the cost of only 3–7 USD per test.

Furthermore, efforts have also been made on multiplexing of μPAD to simultaneously detect targets, which are always challenging, and most

**Table 1**
Paper-based microfluidic analytical devices (μPADs) with different substrate and fabrication methods.

| Hydrophilic substrate | Fabrication methods | Targets | Pathogens | Application | Samples | Linear range | LOD | Refs. |
|---|---|---|---|---|---|---|---|---|
| Filter paper | Wax printing and cutting | virulence factor | *Pseudomonas aeruginosa* | Biomedicine | human saliva and contaminated surfaces | 50–1000 nmol $L^{-1}$ | 10 nmol $L^{-1}$ | [31] |
| Whatman No.1 filter paper | Wax printing | Nucleic acid | *Candida* *Aspergillus* *Cryptococcus* | Biomedicine | Clinical samples | 10–$10^4$ CFU $mL^{-1}$ | 4.90 CFU $mL^{-1}$ *Candida* 4.13 CFU $mL^{-1}$ *Aspergillus* 6.95 CFU $mL^{-1}$ *Cryptococcus* | [32] |
| Whatman No. 1 filter paper | Wax printing | Nucleic acid | HBV | Biomedicine | Buffer | 50 pM-100 nM | 1.45 pM | [23] |
| Whatman No. 1 filter paper | Screen printing and wax printing | Antigen | SARS-CoV-2 | Biomedicine | clinical nasopharyngeal and throat swab samples | 0.1 pg $mL^{-1}$ to 500 ng $mL^{-1}$ | 2.0 fg $mL^{-1}$ | [33] |
| Nitrocellulose paper | Wax printing | Protein biomarker | *Helicobacter pylori* | Biomedicine | Human stool sample | – | $10^4$ CFU $mL^{-1}$ | [34] |
| Carboxymethylcellulose paper | – | Antigen | *Klebsiella pneumoniae* | Biomedicine | Hospital urine samples | – | 0.005 mM | [35] |
| Fusion 5 paper | Wax patterned | Nucleic acid | *Colletotrichum truncatum* | Environmental | Chili pepper | 0.5–75 nM | 232 pM | [36] |
| 210 GSM sheets | Screen printing | Antigen | *Listeria monocytogenes* | Food | Milk and Cheese | 10–$10^8$ CFU $mL^{-1}$ | 10 CFU $mL^{-1}$ | [37] |
| Nylon@rGO/MWCNTs composite paper; Whatman grade 1 chromatography paper | Cutting, screen printing, immersed in melted sliced paraffin | Nucleic acid | SARS-CoV-2 | Biomedicine | Buffer | 25 – 2.5 × $10^{10}$ copies $mL^{-1}$ | 25 copies $mL^{-1}$ | [38] |
| -1Whatman cellulose chromatography paper (grade 1 Chr sheets) | Wax printing | Protein and exotoxin | *S. aureus* *P. aeruginosa* | Biomedicine | Buffer | 0.3–30 ng $mL^{-1}$ | 0.2 ng $mL^{-1}$ and 0.1 ng $mL^{-1}$ | [39] |
| Whatman 3 MM CHR chromatography paper. Whatman Grade 1# filter paper Nitrocellulose membrane HF180 | Wax printing | Nucleic acid | *Escherichia coli* | Food | Juice and milk | $10^3$–$10^7$ CFU $mL^{-1}$ | 1000 CFU $mL^{-1}$ | [40] |
| Whatman #4 chromatography paper | Photolithography | Nucleic acid | *Neisseria meningitidis* | Biomedicine | Human whole serum | 6 -6 × $10^6$ copies | 6 copies | [41] |

studies utilise physical separation of reaction and detection chambers of different analytes to achieve. Rebound et al. [22] reported a multiplexed sensor for malaria detection, with studies performed in only one finger prick of whole blood. They conducted individual diagnoses in village schools in Uganda with tests completed in 50 min, and 98% infected individuals were successfully detected when compared to the gold standard method – quantitative PCR. Somvanshi and co-authors [44] moved one step forward, by integrating machine learning method with their reported multiplexed aptamer-based pathogen detecting μPAD, and therefore allowing for more specific quantification on bacterial load of target pathogens.

It is reported that μPADs suffer from low signal-to-noise ratio especially during the baseline period of a real-time monitoring process of an assay. Various methods have been studied to solve this issue, including chemical modification of cellulose paper substrate, or change of other paper substrate materials, improved digital signal analysis, and employment of novel design of μPAD. Fu and Liu [49] reported that potassium periodate ($KIO_4$)-modified cellulose paper performed the best with a 53% increase in the signal output and a 59% decrease in background noise of the colorimetric ELISA, and only 13% bioactivity loss after the 30 days storage. As for substitution for cellulose paper material, Hao et al. [50] proposed that polyester fibre film DL42 showed the lowest background fluorescence while maintaining strong binding of fluorescent CdTe QDs and RhB, which are the reporters of the assay. Data analysis is also vital to minimising background noise, and Chen et al. [51] demonstrated an advanced laminated device employing red emitting copper nanocluster and red-green-blue (RGB) digital analysis, and the device signal-to-background ratio and the calibration sensitivity were highly improved, together with the calibration sensitivity, limit of detection, and coefficient of determination. Tong et al. [52] showed that 3D threaded μPAD offered a background-free and visual detection of organophosphorus pesticides even in practical sample testing. Examples of μPADs integrating colorimetric, fluorescent, chemiluminescent, and electrochemical detection are summarised in Fig. 2.

Overall, μPAD offers a promising, user-friendly solution for in-field, cost-effective, rapid, sensitive, and specific pathogen detection, eliminating the need of extra equipment and power supply. With the increasing concern caused by the need of pathogen detection in resource limited settings such as low- and middle-income countries (LMICs), μPAD received considerable attention. A variety of substrates has been evaluated, as well as a bunch of fabrication methods for construction of the hydrophobic barriers. Optical and electrochemical detection approaches have been integrated to various bioassays immobilised on the μPAD to satisfy different needs. And the development of μPAD is further accelerated for improved performances with the advancements of technologies in related areas e.g., nanomaterial.

## 3. Pathogen detection methods

As aforementioned, pathogen places a heavy public health burden worldwide, and it is vital to develop reliable, sensitive, and specific detection methods. Conventional pathogen detection is generally divided into three major streams, phenotypic culture-based detection, spectroscopic methods (e.g., Raman spectroscopy), as well as molecular approaches including NAATs (e.g., PCR) and immunological assays (e.g., ELISA).

### 3.1. Culture and spectroscopic methods

Culture methods refer to *in vitro* pathogen growth on specific nutrient media, and they are regarded as the most straightforward approach for identification via visualisation: microorganisms multiply on culturing media to form colonies that may vary in morphological features such as size, shape, and colour, especially when determining between fungal and bacterial genus [56]. In addition, agar media has been further developed for improved culture performance in identification and ease of operation. Chromogenic agar media (CAM) utilised (Fig. 4a) the chromogenic enzyme substrate that can specifically anchor pathogen species due to their distinguished enzymatic activity [57]. It avoids the need for polymicrobial culture since only particular colony colours will be generated by the binding between chromogenic enzymes and pathogens. This method has been widely used in clinical, food and environmental testing since 1990 [58]. For example, Fisher et al. [59] modified MYCOn™ culture media which is used to detect *nontuberculous mycobacteria* (NTM), for extensive pathogen detection, as standard media with Mycobacterium genus colonies will inhibit the growth of

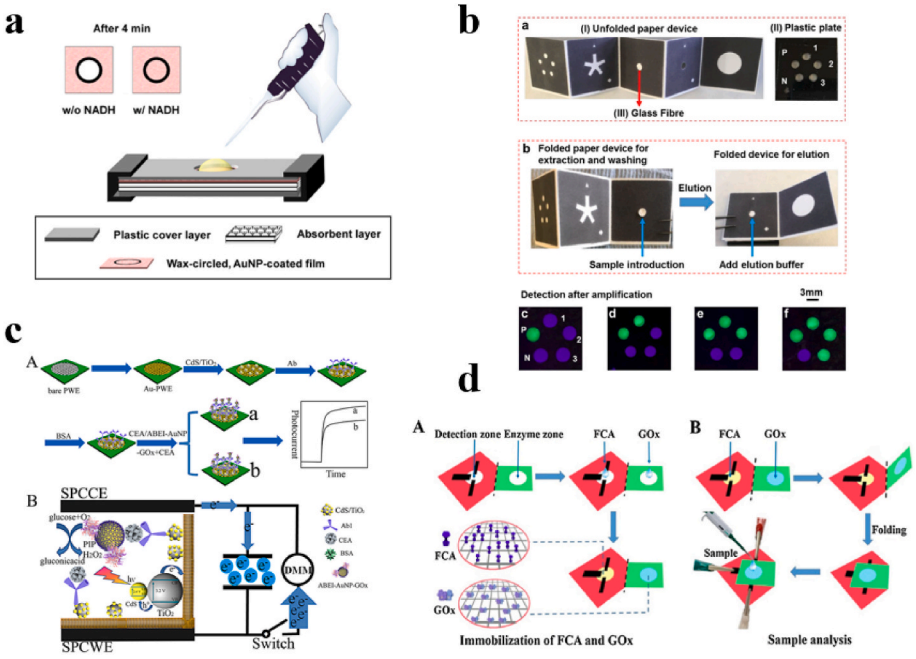


**Fig. 2.** Examples of μPADs integrating colorimetric (a), fluorescent (b), chemiluminescent (c), and electrochemical (d) detection. Reprinted from Refs. [42,53–55], respectively.

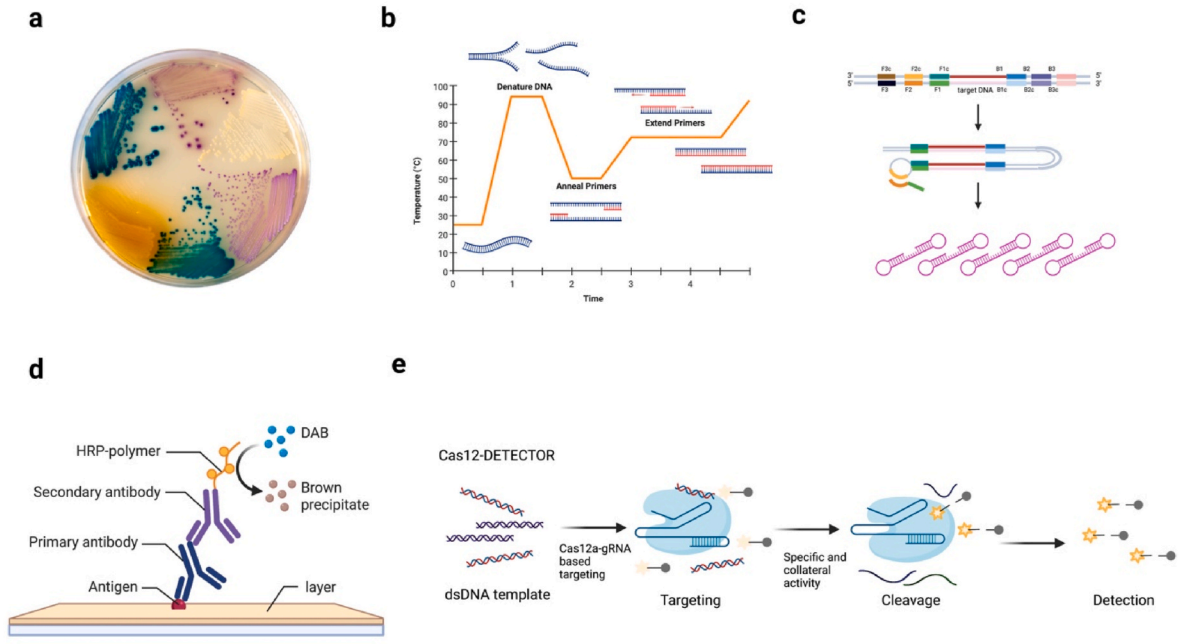


**Fig. 3.** Schematic of state-of-art detection approaches. A, A top view of a multiple pathogen chromogenic culture from CHROMagar™ Orientation. b, PCR workflow. c, Principle of LAMP and uses six specific primers to recognize discrete regions of the target DNA. d, μsandwich ELISA reaction mechanism that a primary antibody recognizes an antigen, and a specific secondary antibody conjugated with horseradish peroxidase. e, Cas12-based nucleic acid detection.

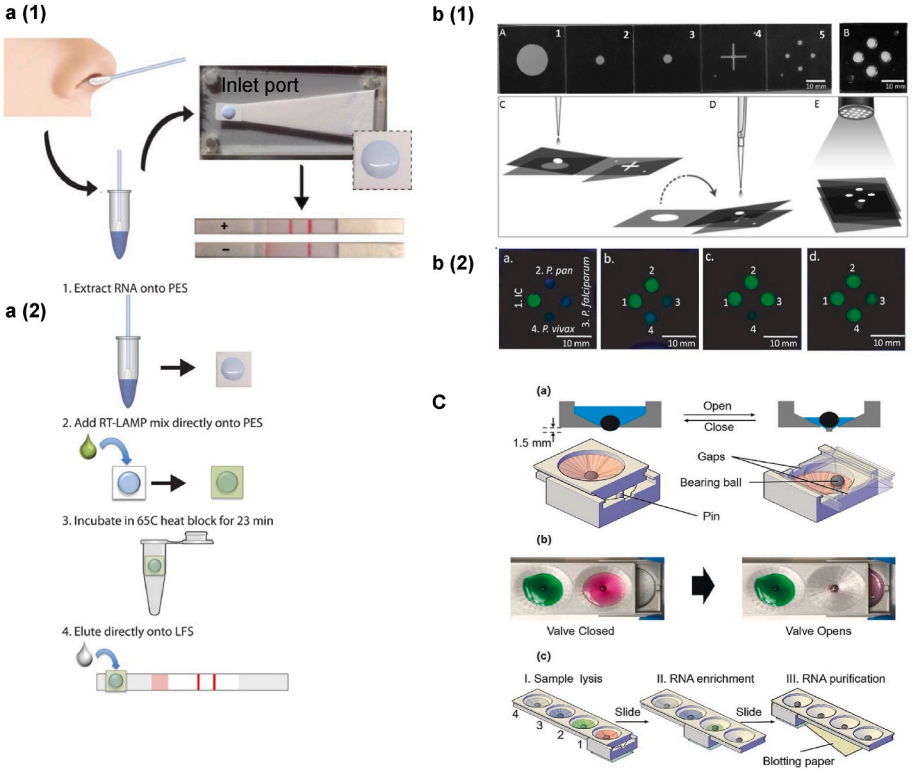


**Fig. 4.** Brief illustration of a published paper-based device for NAT. a Polyethersulfone (PES) filter paper-based RNA extraction device was developed for one-step cell lysis and RNA extraction. A (1) shows the top view of the device, it consists of an inlet port, absorption μPAD and acrylic fixture (shown in the background); a (2) explains the workflow of how the detection is undertaken [119]; b (1), Paper-based multiplexed LAMP detection of malaria in blood. b (2) Results of multiplex LAMP amplification under UV light. Under UV excitation, green calcein emission occurs in the presence of pyrophosphate [146]. c, Ball-valves in the buffer unit and their use for sample preparation[121]. All figures are reprinted (adapted) with permission from Analytical Chemistry. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Gram-positive bacteria. They improved the media by employing low pH and sodium dodecyl sulphate (SDS) to remove decontamination, and they detected *nontuberculous mycobacteria* (NTM) in various water matrices with a higher referencing standard.

Although this method possesses advantages such as low cost, ease of operation, and pathogen quantification, it lacks sensitivity that may hinder its use in samples with extremely low target concentrations. Besides, various factors e.g., complicated matrices with inhibitors, can affect the pathogen growth, which may lead to false negative results. Finally, multiple steps including pre-enrichment, selective enrichment, plating on selective media, and then biochemical or serological confirmatory tests are required for definite identification of pathogen with a turn-around time of 2–3 days, which is time- and labour-intensive, and therefore is unable to give a rapid testing result [60].

Spectroscopic methods have been widely applied in pathogen detection especially in food safety. Common techniques include infrared (IR) spectroscopy and Raman spectroscopy, which identify pathogens via comparison between sample 'fingerprints map' and molecular characteristics of specific peaks which relate to the composition of cell walls. Both IR and Raman spectroscopy depend on inelastic scattering of excitation light and molecular resonance to obtain 'fingerprints map'. While IR is not suitable for samples in solutions and has a narrower detection range and less specific and less readable results compared to Raman spectroscopy, the latter is far less used due to its weak signals, fluorescence interference and unaffordable equipment [61]. To solve this issue, surface-enhanced Raman spectroscopy (SERS) was developed, combining merits from both IR and Raman spectroscopy. Tests can be done on solutions, fluorescence interference is suppressed, and Raman signal can be enhanced to $10^7$ to $10^{14}$ orders of magnitude by integration of noble-metal nanoparticles [62]. Featured by its high efficiency, sensitivity, and stability, SERS has been popular in pathogen detection in various fields.

### 3.2. Molecular method

Contrary to conventional culturing, molecular methods is advantageous in rapid, sensitive and specific detection. PCR (Fig. 4b) is the gold standard of molecular pathogen detection and is capable of quantification [63]. Variations of PCR methods have been developed in the past decades, including real-time PCR, reverse-transcript PCR (RT-PCR) and multiplex PCR [64], improving its performance for faster and more sensitive and specific detection on multiple pathogen targets. Multiplex PCR is especially important for pathogen detection in environmental settings. In 2019, Sato et al. [65] developed using environmental DNA (eDNA) metabarcoding for detecting the pathogenic Leptospira and related microbes in aquatic samples. They used 4 pairs of primers to generate PCR products for specific high-throughput sequencing, and they compared the sequencing results finding the microbiota in each river shifted over time, especially in northern Okinawa, Japan. This eDNA multiplex method identifies that leptospiral survival and the persistence environment depend on interaction with other microbes, which highlights the importance of tracing microbial lineage in the environment to predict the pandemic area.

Although PCR presents a reliable and robust approach for pathogen detection, most experiments are conducted in a laboratory setup with an expensive equipment, which is less accessible for LMICs. Recently, isothermal amplification (Fig. 4c) such as loop-mediated isothermal amplification (LAMP) [66], recombinase polymerase amplification (RPA) [67], rolling circle amplification (RCA) [68] has been developed to address these challenges, as an alternative way with less central laboratory equipment. They significantly reduce the complexity of reaction conditions, especially eliminating the need for thermal cycling, offering a great opportunity for POC/POU testing. However, NAATs can suffer from false positive results especially when the primers are not properly designed, or from non-specific detection, which include fluorescent DNA intercalating dye and turbidity (for LAMP where pyrophosphate precipitate during reaction), and they cannot distinguish spurious amplicons, leading to false positive results [69] Besides, there are concerns on carry-over contamination if the reaction container is not properly sealed. Aerosols with high concentration of amplified products and/or template sequence can contaminate surrounding areas and samples, leading to false positive results [70]. To solve the problem, optimal primer design and fluorescently labelled primers [69,71,72] are shown to improve the specificity of the assay, and the introduction of CRISPR/Cas technology on sequence-specific cleavage [73] is also proven to be effective solutions to the false positive results. In addition, chemical additives such as tetramethylammonium chloride (TMAC) [74] are also proven to improve assay specificity by inhibiting non-specific amplicon formation. As for elimination of carryover contamination, approaches including employment of uracil-DNA-glycosylase (UNG) [75], restriction endonuclease Gsu I [76], CRISPR/Cas9 enzyme [77], and Cod-uracil-DNA-glycosylase [70].

Other than nucleic acid amplification, immunological detection shares valuable strategies to carry out pathogen detection and further quantification. The mechanism mainly for testing a certain pathogen is based on the specific antigen–antibody interactions [78]. It is quite a way of understanding the host immune conditions. There are various forms of immunological methods, including latex angulation [79], immunofluorescence assay [80] and enzyme-linked immunosorbent [81]. Fig. 4d illustrates the detection principle of common sandwich ELISA. Though those assays could not meet the requirement of real-time detection, they are significant for the study of pathogen interaction with the host and assessment associated with pathogen infection.

With modern development of biotechnology, the emergence of CRISPR/Cas system in pathogen detection is gaining wide attention (see Fig. 3). Relying on its ability to recognize and cleave specific DNA and RNA sequences, this system has adapted and coupled with well-developed systems to enhance their performances. Generally, CRISPR/Cas systems work by recognising specific sequence and triggering a cleavage, which creates a DNA double-strand break (*cis*-cleavage) [82]. Different Cas proteins own different features, and the most renowned ones are Cas9, Cas12, Cas13 and Cas14. Credits to the trans cleavage activity (i.e., non-specific single stranded DNA cleaved when cis cleavage presents) of Cas12a, it is the most popular CRISPR/Cas detection system with higher sensitivity and specificity [83]. Model cas12a platforms include DETECTR (DNA endonuclease targeted CRISPR *trans*--reporter, Fig. 4e) [84], HOLMES (1-h low-cost multipurpose highly efficient system) [85], and CDetection (Cas12b-mediated DNA detection) [86]. During the Covid-19 pandemic, DETECTR drastically reduced the detection time of beta coronavirus severe acute respiratory syndrome (SARS)-CoV-2 coupled with RT-LAMP by 40 min. RT-LAMP ease the burden of RNA extraction and amplification, ssRNA with customized FAM and biotin would be cleavage generating yellow fluoresce when binding with specific amplified SARS-CoV-2 segments. This assay provides a visual alternative compared to real-time PCR, and it is much more sensitive than LAMP itself, which could reach 95% positive predictive agreement and 100% negative predictive agreement.

To sum up, phenotypic culturing has been the gold standard of pathogen detection, presenting a simple and straightforward method but it is time- and labour-intensive, limiting its potential to be adapted for rapid field-testing. The emergence of molecular methods offers a rapid alternative, further providing understanding on underlying genetic mechanism. Besides, it is capable of processing multiplexed samples. However, molecular diagnostics usually require laboratory setups, and the equipment is generally not affordable in LMICs. For example, a simple PCR machine like Bio-Rad T100 thermal cycler is priced at 4912 $, while a quantification model could be over 90,000$ [87]. The development of isothermal amplification eliminates the need for expensive equipment, but they are also suffered from false positive issues even when coupled with specific detection of Cas12 systems [88]. Therefore, more advanced pathogen detection platforms are urgently calling to be explored. An example of different biosensors for the detection of pathogens are summarised in Table 2.

## 4. Paper-based microfluidic analytical devices enabled field-testing of pathogens

Monitoring pathogens onsite is vital for public health, as infectious diseases cause more than 1.2 million deaths each year in low- and middle-income countries (LMICs) [95]. It is therefore of great importance to develop simple, low-cost, robust, portable, sensitive, and specific POC/POU devices for early diagnostics and surveillance of pathogens. World Health Organisation (WHO) [96] established guidelines of the ASSURED criteria, affordable, sensitive, specific, user-friendly, rapid, and robust, equipment-free and deliverable, to assess the performance of POC/POU devices. Under this situation, μPAD

**Table 2**
Examples of the different biosensors for the detection of pathogens.

| Biosensor | Transducer type | Applications | Results | Refs |
|---|---|---|---|---|
| Bioluminescent | Optical | Quantification and detection of heavy metals, food toxicants, and environmental monitoring | assess the uptake and survival of bacteria within the freshwater | [89] |
| Colorimetric | Optical | Water- and foodborne pathogens | LOD of 8.5 and 1.3 ng $mL^{-1}$ for norfluoxetine and BDE-47, respectively. | [90] |
| Sandwich ELISA | Electrochemical | salmonella in meat samples | 5 h incubation in 1–10 cells 25 $g^{-1}$ | [91] |
| QD nanosensor | Fluorescence | rAAV | N/A | [92] |
| Cross-linking with GA | Piezoelectric | The organophosphorus neurotoxin in the water | 50 mg $m^{-3}$ | [93] |
| SPR | Optical | CymMV | 1200 pg $mL^{-1}$ | [94] |

Abbreviations: LOD: Limit of detection; ELISA: Enzyme-linked immunosorbent assay; rAAV: recombinant adeno-associated virus; CymMV: Cymbidium mosaic virus; SPR: Surface plasma resonance; N/A: No information.

stands out as an ideal onsite detection platform owing to its cost-effectiveness, readily stored and transported ability, and biodegradable and compatible feature. Besides, compared to other devices, μPAD require minimal fabrication and extra-equipment and energy, greatly simplify the operation procedure and is therefore user-friendly, eliminating the need of central laboratory setup and trained personnel. For example, Reboud et al. [22] proposed a low-cost, rapid, and multiplexed μPAD for accurate and sensitive malaria detection. They used a paper origami to extract and enrich DNAs from samples, amplified target products in a chamber with pre-loaded LAMP reagents, and finally employed a lateral flow assay sealed inside a transparent cassette for detection. This whole procedure can be completely conducted for onsite detections, and the authors indeed carried out experiments in a school in Uganda. In addition, He et al. [97] reported a paper-sensor for infection testing that can be used by unskilled users in a low resource setting. The paper sensor has three layers, with a bottom layer containing chromogenic agar for permissive bacterial growth, a middle layer made of cellulose paper with an inlet port for sample introduction, and finally, a top layer with an array of laser-patterned wells containing different antibiotics in different doses for the susceptibility testing. Once the device is fabricated and integrated, users only need to peel off a small sealing tape at the inlet port, load sample on it, and finally seal the device to avoid evaporation. As a result, μPAD has been popular in onsite pathogen detection and the past decade has witnessed its thriving development.

### 4.1. Organism detection

Pathogen culturing refers to the multiplication of microorganism cells on a culturing media under controlled laboratory conditions. It includes a series of operations such as pre-enrichment, selective enrichment, selective plating, biochemical screening, and serological confirmation. Viable microorganism is therefore isolated and enumerated. Culturing has been therefore regarded as the gold standard of clinical pathogen detection, while they are demanding in laboratory setups and trained personnel [98].

With the advancement of modern technologies such as microfluidics and nanomaterials, there are opportunities that allow pathogen to be cultured and rapidly detected in a resource limited setup. Briefly, there are two types of nanomaterial-based detections. The first one utilises the localized surface plasmon resonance (LSPR) of noble metal nanoparticles such as gold nanoparticles (AuNP) and silver nanoparticles (AgNP), as they possess visible colours that can be employed as colorimetric reporters especially in the LFA. For example, Lu et al. [99] proposed an aptamer-based lateral flow test strip for simultaneous detections of *Salmonella Typhimurium*, *Escherichia coli O157:H7*, and *Staphylococcus aureus*. AuNPs were employed as the signal probe and the visual limits of detection of the strip within 10 min were $10^3$ CFU $mL^{-1}$, $10^4$ CFU $mL^{-1}$, and $10^4$ CFU $mL^{-1}$, respectively. Since LSPR extinction peak is affected by the local refractive index change and self-assembly of the nanoparticles, for example, when aggregated, AuNP presents to be purple while suspended it is red, and biosensors have been developed based on this property [100]. The other type of nanomaterial-based detection is nanoenzyme. Nanozymes are a type of artificial enzymes with higher stability in harsh conditions, cost-effectiveness, ease of mass production, and highly tuneable catalytic activities [101]. To date, a variety of nanoenzymes are reported, including metal oxide nanoparticles, nanoclusters, quantum dots, carbon nanostructures, nanowires, and composites such as organic-inorganic hybrid nanoflowers and metal-organic frameworks [102–104]. Studies have also demonstrated their use in on-site pathogen detection with enhanced analytical performance and robustness. Jiang et al. [105] proposed a novel immunochromatographic assay (ICA) with enhanced sensitivity for the visual and quantitative detection of *E. coli* O157:H7. Pt–Au bimetal nanoparticles (NPs) were accumulated on the test zone and by employing its high peroxidase activity toward 3,3′,5,5′-tetramethylbenzidine, characteristic-coloured bands were formed, which enabled visual detection of *E. coli* O157:H7 without instrumentation. Strong visible colour change in less than 1 min in low concentration range of *E. coli* O157:H7 was observed, and quantification was performed using a commercial assay meter. Results indicated that the sensitivity was improved more than 1000-folds compared to the conventional test strip based on coloured gold-colloids. Guo et al. [106] proposed an intelligent adhesive tape for the development of a "three-in-one" platform for rapid sampling, photo-controlled release, and SERS detection of pathogens from infected wounds. *Pseudomonas aeruginosa* and *Staphylococcus aureus* were employed to demonstrate the performance of the platform by pasting the tape on a skin burn wound. An analytical time of only several hours were needed for the early growth of pathogens, indicating its great potential as a POC testing device for health care. Yuan et al. reported [107] a handheld and low-cost μPAD based on an immunoassay integrating fluorescent nanomaterials, including graphene quantum dots (GQDs) and gold nanoclusters (AuNCs). The nanomaterials are conjugated with antibodies to convert pathogen presence to a colourful fluorescence signal. This device is able to detect multiple analytes simultaneously with an LOD of subnanogram/mL and a reaction time of just 10 min.

In addition to generic pathogen detection, its application on monitoring of antimicrobial resistance (AMR) is an important branch of pathogen detection. AMR refers to a condition where microorganism once susceptible to antimicrobial reagents are no longer sensitive to them. AMR is defined as a broad term including antibiotic (for bacteria), antiviral (for viruses), and antifungal (for fungi) resistance. It has been a global public health threat, leading to 1.27 million deaths each year [108]. And it also puts a heavy economic burden on the society – 1.5 billion euros have been attributed to AMR per year just within the EU. It emerges at an alarming rate: penicillin was first prescribed to patients in 1941 while the first reported case of penicillin resistance was in 1942. Accurate identification of antimicrobial resistant profile of microorganisms is therefore of critical importance on public health.

He et al. [97] for the first time proposed a laser-patterned paper--based device for simultaneous culturing and detection of *Escherichia coli*

together with multiple antibiotic resistance testing, which is noted by a visible colour change. This device is composed of three layers, the bottom layer is a miniaturised culture base where bacteria grow, the middle layer allows for sample inlet and distribution, while the top layer is where antimicrobial susceptibility testing (AST) is performed, and the multiplex testing is achieved by multiple test zones noted by laser-direct-write polymer walls. Compared with conventional agar-plate based disk diffusion susceptibility testing, this device shows the potential to be further developed into a POC device to tackle the global challenge of antibiotic resistance. Punjabi et al. [109] established a core-shell nanoparticle fabricated paper-based POC devices of 100% sensitivity and specificity to detect resistance in penicillin, cephalosporin and carbapenem via a visible colour change within 30 min and the LOD is $10^5$ CFU/mL. The core is composed of chitosan nanoparticles of size around 15 nm, which are coated with starch-iodine indicator with a final size of 47 nm. When ARO is present, these nanoparticles will change from blue to white. Having tested this device in complicated matrices involving common confounding factors without a sign of interference, it shows excellent promises to be further developed into a screening device for known antibiotic-resistant bacterial infections. Sun et al. [110] presented a portable paper-based band-aid (PBA) utilising different visible colours to indicate *Escherichia coli* infection (yellow) and drug resistance (red) within 4 h with a LOD of $10^4$ CFU/mL. This is achieved as they employed the acidic bacterial microenvironment nature for drug-sensitive (DS) bacteria. Bromothymol blue (BTB) was immobilised onto one band, which would change from green to yellow under acidic condition, marking the presence of DS bacteria. On the other hand, antibiotic-loaded nanomaterial was coated with chitosan to attract negatively charged bacteria. Then the antibiotic released in response to the acidic environment, killing the DS bacteria, leaving only the drug-resistant (DR) bacteria progressed to the next band. As the resistance indicator, Nitrocefin would change from yellow to red when encountering beta-lactamase generated from DR bacteria. The resistance-detection band also incubated with PCN-224, a porphyrin-base metal-organic framework which would produce ROS under the irradiation of light. ROS would significantly damage the bacteria and weaken the resistance, suggesting the efficacy of photodynamic therapy (PDT) in treating antibiotic resistance. In addition to colorimetric detection (including visible colour change detection), phenotypic testing can also integrate other techniques, for example, electrochemical detection and surface-enhanced Raman spectroscopy (SERS). Hilton et al. [111] proposed a paper-based SERS sensor to directly measure the ESBL activity for phenotypic differentiation among ESBL-Escherichia coli, narrow-spectrum BL ones and non-resistant ones. Two different colorimetric reporters were used, CENTA and Ceftriaxone, which would be hydrolysed by beta-lactamase of different reactivity and corresponding SERS barcodes will be released with sulphur for SERS detection. Recent advances in portable Raman instrumentation allows for improved sensitivity and spectral resolution, and therefore higher density multiplexing might be achieved. This work could also be further expanded to a library with other beta-lactams by synthesising additional beta-lactam starting material representing the drugs and more Raman barcodes.

Wang et al. [112] developed a dual aptamer μPAD for the simultaneous detection of Acinetobacter baumannii (AB), *Escherichia coli* (EC), and multidrug-resistant *Staphylococcus aureus* (SA) in 35 min. Aptamers specific to its target bacteria were immobilised onto the paper substrate via UV cross-linking and blocking before bacteria were incubated. Then a second aptamer-biotin conjugate was applied, followed by HRP-streptavidin and tetramethylbenzidine (TMB). A visible blue colour change will be observed once the bacteria are present. The LOD was roughly determined by naked eye via 10-fold serial dilutions, and the results showed that LODs of AB, EC and SA was $10^3$, $10^4$, and $10^5$ CFU/μL, respectively. Another example is that Pang et al. [113] proposed a paper-based ELISA method to detect *Escherichia coli* O157:H7 within 3 h. In this study, the sample was immobilised on chitosan-glutaraldehyde pre-treated filter paper before blocked with BSA. The first antibodies then bound to the sample, followed by subsequent binding of HRP-labelled second antibodies. After colour development with TMB, pictures were taken and analysed using the software ImageJ for quantitative results. The LOD was determined to be $10^4$ CFU/mL.

### 4.2. Nucleic acid detection

Nucleic acid testing (NAT) has been widely operated with reliable sensitivity and specificity, in particular, NAATs contributes the majority of research [114]. With the increasing demands of applying NAT in the field, μPAD provides a portable detection platform for sample processing and target detection.

#### 4.2.1. Sample processing with μPADs

Paper demonstrates a sustainable physical deposition of the coating and adhering by modifying with materials such as nanomaterials or polymers [115]. Coated with nanoparticles created imperfections on paper, which increases the interaction on the surface. A reasonably successful commercial product for storing and extracting nucleic acid is FTA card® (GE Whatman, Maidstone, Kent, United Kingdom). The card matrix can subsequently secure nucleic acids with reagents that promote cell lysis and protein denaturation and stabilize them at room temperature [116]. It was initially applied to medical blood samples only and was widely used in downstream molecular testing. A recent study by Birnberg et al. [117] used a honey-baited FTA card to pool mosquitos' saliva in field conditions to detect arthropod-borne viruses. Though follow-up analysis experiments were conducted by real-time PCR and NGS, the pilot of using the FTA card indicates a valid approach for sample storage and preparation, which provides an alternative for in-field surveillance.

#### 4.2.2. Nucleic acid detection with μPADs

As aforementioned, NAATs have been regarded as the most popular approach in nucleic acid detection. Among all amplification methods, PCR not only remains to be the gold standard, but also is capable of integrating with μPADs. Lee et al. [118] reported a novel paper-based capillary electrophoresis (pCE) microdevice that can differentiate single-stranded DNA (ssDNA) with 4 bp resolution in a 2.9 cm-long CE separation channel. They successfully demonstrated the identification of the PCR amplicons of two target genes of *Escherichia coli* O157:H7 (rrsH gene, 121 bp) and *Staphylococcus aureus* (glnA gene, 225 bp) with the employment of two bracket ladders (80 bp for the shortest and 326 bp for the longest) and the reaction was completed within 3 min using the relative migration time ratio without effect of the CE environments.

In addition to PCR, isothermal amplification is flourishing in field-testing of pathogens, as it is more compatible with resource limited setups. An integrated device could therefore be developed with sample processing and detection both done on the single device. Rodriguez et al. [119] developed a paper-based that allows *in situ* RNA extraction and amplification. Coupled lateral flow strip made it a one-step device for detecting Influenza A (H1N1) with immediate results (Fig. 4a). The whole assay is completed in 45 min with a 10-fold sensitivity improvement compared to previous assays. Customized design will expand the detection scope, Xu et al. [146] integrated LAMP into a "manual machine" that allowed a multiplexed three taxon-specific test plus control for measuring malaria in human blood. Fig. 4b presents a 5-panel design that contains extraction and elution via folding. A UV lamp visualizes the reaction in the chamber with compatible identifying Plasmodium diagnosis accuracy, and only P. falciparum was sensitivity below 80%. Zhuang et al. [120] proposed a SERS-based CRISPR/Cas assay on μPADs for supersensitive detection of pathogenic bacteria in foods. RPA was employed for target gene (i.e., invA gene) amplification while ssDNAs were designed to interlink SERS nanoprobes. With cas12a recognising target amplicons, *trans*-cleavage activity was activated to

shred linker ssDNA and thus the nanoprobes aggregated. The degree of aggregation reflected the concentration of *Salmonella typhimurium (S. typhi)*, which was determined by the proposed μPADs and monitored by a Raman spectrometer. The limit of detection for *S. typhi* was approximately 3–4 CFU $mL^{-1}$ for spiked milk and meat samples with a dynamic detection range from 1 to $10^8$ CFU/mL within 45 min.

Applications relevant to environment testing are limited since deploying them fully in the field is still troublesome. One study features Zika virus (ZIKV) virus detection in human urine, saliva, and local water with a paper-based unit for RNA enrichment and purification [121]. The significance of this device is that they wisely coated chromatography paper underneath the valve to hold the treated sample for subsequent steps (Fig. 4C). They tested this paper in comparison with other familiar options like FTA card or glass fibre, and it was chosen for its best performance in ZIKA. With this slide design, contamination is vastly reduced for each step before the amplification. Their detection limit is 0.5 PFU of ZIKV in urine and saliva samples and 0.1 PFU of ZIKV in water samples, and hopefully, this device could be used in broader field testing in the future.

There is an emerging trend of nanomaterial modified μPAD for enhanced performance of pathogen detection. And there is a number of studies which target nucleic acids rather than microorganisms [122]. An example is that Zhou et al. [123] proposed a simple, low-cost, and universal gold nanoparticle (AuNP) aggregation-induced photothermal biosensing platform for the visual quantitative genetic detection. *Mycobacterium tuberculosis* (MTB) DNA was used as a model target with a LOD of 0.28 nM within 40 min.

Paper-based devices for nucleic acid is still not widely commercialized in the market. It can be found that commercially available products were in stagnation within a decade. Regarding those introduced to the in-house paper-based device, the associated design is expected to be quite sophisticated for nucleic acid detection. This kind of detection requires several steps prior to the final detection. Therefore, each design is unique based on the detection taxon.

### 4.3. Chemical biomarkers relative to pathogen detection

In addition to phenotypic pathogen culturing and nucleic acid testing, there are indirect methods which measures chemistry representing pathogens such as metabolites and toxins (see Fig. 5) . Adenosine triphosphate (ATP) is the universal energy molecule supporting living activities and is only present in living cells [124]. Therefore, it has long been employed as a measure of living microbe existence for quantification of pathogens since 1990s [125,126]. In 2015, Jin et al. [127] reported a cost-effective special Z-folding designed μPAD for *Salmonella* live cell detection via ATP quantification, and the limit of detection (LOD) was 1 μM for ATP detection and $2.6 \times 10^7$ CFU/mL, indicating its great potential for POC application.

While ATP represents a great practice due to its wide presence in living cells, it lacks the ability to detect specific pathogen, and therefore more is to be explored on improving the specificity. To solve this problem [128], reported a portable and cost-effective μPAD for cultivating bacteria *in situ* and rapidly testing for nitrite on the same device. The enzyme β-glucuronidase, specifically produced by *Escherichia coli*, converted the pre-immobilised 5-bromo-4-chloro-3-indolyl-β-D-glucuronide sodium salt (X-GlcA) (a colourless substrate) to a blue colour, and nitrite detection was based on the principle of the Griess reaction with a linear detection range of 0–1.6 mg/dL ($R^2 = 0.989$). The proposed μPAD can quantify pathogen concentration in the range of $10^4$–$10^7$ colony forming units (CFU)/mL within 6 h. Other than enzymes, toxins secreted by specific pathogens have been extensively used as biomarkers, because their presence imply bacterial presence and indicate bacterial virulence and pathogenicity. For example, pyocyanin (PYO), proven to be specifically produced by *Pseudomonas aeruginosa* (PA) [129], has been employed as a biomarker for rapid detection of this common waterborne pathogen [130], In addition, epsilon toxin (ETX) [131], shiga toxin (Stx) [132], and Yersinia stable toxin (Yst) [133] have been employed for the detection of *Clostridium perfringens*, *Escherichia coli*, and *Yersinia enterocolitica*, respectively. Silva et al. [134] reported a

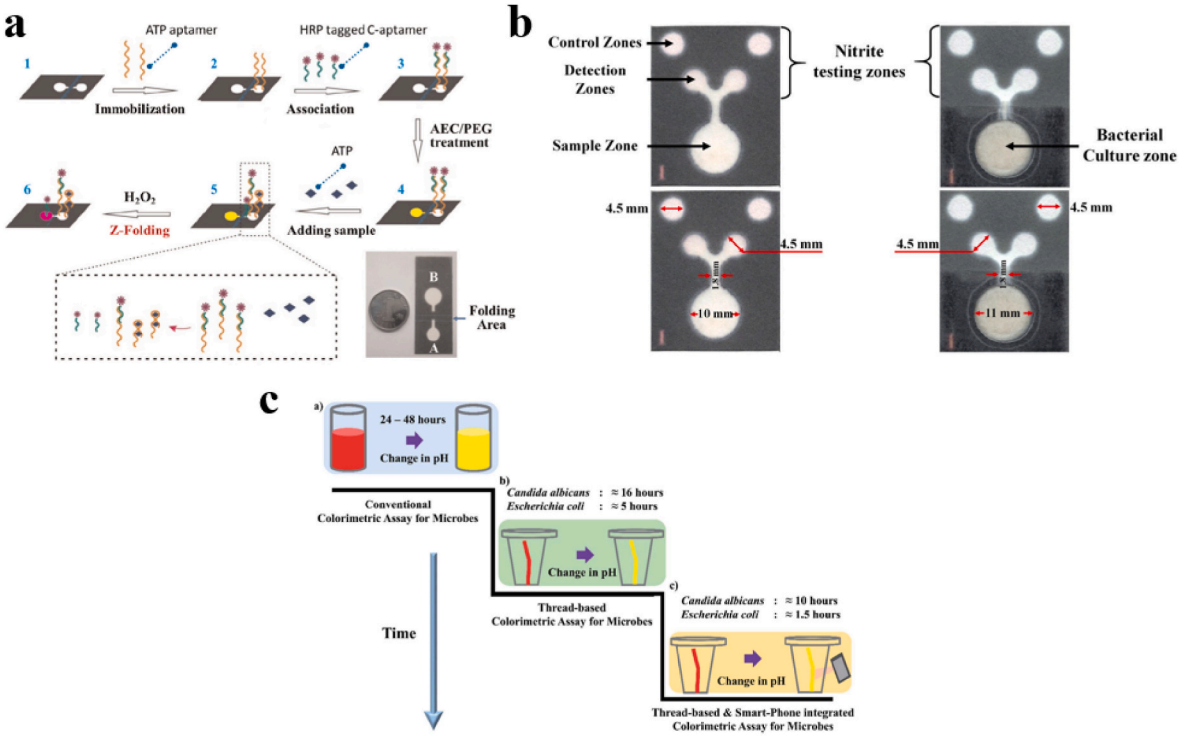


**Fig. 5.** *Schematic illustration of (a) the cost-effective Z-folding controlled liquid handling microfluidic paper analysis device for pathogen detection via ATP quantification. (b μPAD for nitrite quantification (c) colorimetric test (a) conventional (b) thread-based device (c) thread-based device and smart-phone imaging. Components present: microbial inoculum, sugar media and* pH *indicator.* [127,128,135].

portable, selective, sensitive, inexpensive and disposable electrochemical paper-based analytical device (ePAD) for the detection of PA via PYO in human saliva and contaminated surfaces, without any sample preparation or separation steps. Besides, they also applied the device to monitor bacterial growth by correlating PYO levels with the optical density values of PA colony forming units (CFU). Furthermore, pH-dependent colour change is also frequently utilised in pathogen detection. A commercial multifilament cotton thread was integrated with smartphone-based imaging for pathogen detection [135]. Target pathogens scavenge the sugar-based media components of sugars and release acidic by-products, triggering pH-based colour change (from red to yellow). This method significantly reduced detection and data interpretation time of *Candida albicans* and *Escherichia coli* compared to conventional microbial methods, representing a ready-to-use, low-cost and straightforward technology with applicability in resource-constrained environments.

As aforementioned, profiling antibiotic resistance is a vital application of pathogen detection. Other than phenotypic detection (e.g., antimicrobial susceptibility test) and molecular diagnostics focusing on genes, non-nucleic acid biomarkers can also indicate antibiotic resistance status. Gao et al. [136] established a rapid, quantitative, and high-throughput paper-based device to phenotypically test antibiotic resistance of *Pseudomonas aeruginosa* wild-type PAO1 to gentamicin. This is done by measuring the electrons transferred during the microbial metabolic activities, which are inversely proportional to antibiotic concentrations. The antibiotic resistance could be estimated in 3 h while a conclusive result could be obtained in 5 h.

## 5. Conclusion and perspectives

Infectious diseases have posed a serious threat to global public health and extensive surveillance on pathogens from both clinical and environmental perspectives support timely response to potential disease outbreaks. Conventional laboratory-based detection methods are time- and labour-intensive, besides, they are also hard to adapt into portable devices for onsite detection, especially in resources limited settings. Recent developments on μPADs offer a promising low-cost, rapid, and sensitive pathogen detection tool for in field measurement on pathogen and disease related targets, including microorganisms, nucleic acids, and other chemical biomarkers such as proteins, metabolites, and toxins. Here in this review, we briefly introduced μPAD with its substrate, fabrication techniques, and its critical role in POC/POU application for field testing of pathogens. We also critically reviewed various studies that demonstrate the feasibility of μPAD in pathogen detection from three levels, microorganism-level, gene-level and chemistry biomarker-level, and μPADs employed as case studies in this review are summarised in Table 3. With the advancement in material technology, nanoparticle is playing a significant role for the paper based microfluidic device for in field testing. The implementation of nanoparticle can be briefly summarised to be 1) colorimetric reporter utilising LSPR property of noble metal nanoparticles (e.g., AuNP) 2) nanoenzymes (e.g., quantum dots) that catalyses reactions as a replacement of the traditional enzymes. Both of the methods are prone to improve the analytical performances such as sensitivity and reliability of the device. Although extra steps of modification of the paper device are required to implement

**Table 3**
Summary of μPADs as case studies.

| Hydrophilic substrate | Target | Sensitivity | Application to enhance readability | Refs. |
|---|---|---|---|---|
| nitrocellulose membrane | Salmonella Typhimurium, Escherichia coliO157:H7, and *Staphylococcus aureus* | $10^3$ CFU $mL^{-1}$, $10^4$ CFU $mL^{-1}$, and $10^4$ CFU $mL^{-1}$, | Aptamer conjugated with AuNP for colorimetric readouts | [99] |
| nitrocellulose membrane | *E. coli* O157:H7 | 100 cells $mL^{-1}$ | Peroxidase like property of porus Pt–Au NP for colorimetric readouts | [105] |
| graphene and modified with a synthetic *o*-nitrobenzyl derivative molecule | *Pseudomonas aeruginosa* and *Staphylococcus aureus* | 1.8 nM | gold nanostars as SERS substrates | [106] |
| Whatman cellulose chromatography paper (grade 1 Chr sheets) | *P. aeruginosa* and *S. aureus* (protein A and exotoxin A) | 0.2 and 0.1 ng $mL^{-1}$ | graphene quantum dots (GQDs) and gold nanoclusters (AuNCs) conjugated with antibodies to indicate the immunoassay results | [107] |
| cellulose-based filter papers (CF1) | *E. coli* | N/A | Culturing bacteria before detection | [97] |
| Whatman qualitative filter paper grade 3 | β-lactamase enzyme | $10^5$ CFU $ml^{-1}$ | starch–iodine coated chitosan nanoparticle for colorimetric readouts | [109] |
| sterile cellulose paper | *E. coli* | $10^4$ CFU $mL^{-1}$ | bromothymol blue (BTB) for colorimetric readout | [110] |
| SERS paper sensors patterned with silver nanoparticles (Metrohm Raman) | *E. coli* with various β-lactamase enzyme | $10^5$ CFU | CENTA (a colorimetric reporter for β-lactamase activity) | [111] |
| nitrocellulose membrane | Acinetobacter baumannii, *Escherichia coli*, and multidrug-resistant *Staphylococcus aureus*. | $10^3$, $10^4$ and $10^5$ CFU $\mu L^{-1}$ | TMB as colorimetric reporter oxidised | [112] |
| Whatman No.1 filter paper (Whatman International, Ltd., Maidstone, England) | *Escherichia coli* O157:H7 | $10^4$ CFU $mL^{-1}$ | Image acquisition using scanner or smart phone | [113] |
| a mineral paper layer (140 μm thickness) | *E. coli* O157:H7 and *S. aureus* | $9.3 \times 10^1$ copies (0.5 pg) and $1.6 \times 10^2$ copies (0.5 pg) | Mineral paper composed of calcium carbonate (>75%) and a small quantity (<25%) of non-toxic resin (polyethylene (PE)) for enhanced capillary electrophoresis | [118] |
| cellulose (Whatman GB003 blotting paper, cat# 10426972) absorbent pad | Influenza A (H1N1) | $10^6$ copies $mL^{-1}$ | Isothermal amplification (LAMP) for improved sensitivity | [119] |
| Whatman Grade 4 filter paper | *Salmonella typhimurium* (*S. typhi*) | 3–4 CFU $mL^{-1}$ | The preparation of gold nanostar@4-mercaptobenzoic acid@goldnanoshell structures (AuNS@4-MBA@Au) for SERS signal | [120] |
| Whatman 1 chromatography paper | Zika Virus | 0.5 PFU | Isothermal amplification (LAMP) for improved sensitivity | [121] |
| Whatman No. 1 Chromatographic paper | ATP and Salmonella | 1 μM and 2.6 × 107 CFU $mL^{-1}$. | Z-folding for liquid control and colorimetric readout | [127] |
| Whatman No. 1 filter paper (Cat No. 1001–185) | *E. coli* | $10^4$ CFU $mL^{-1}$ | 5-bromo-4-chloro-3-indolyl-β-D-glucuronide sodium salt (X-GlcA) turns blue upon presence of target | [128] |
| filter paper | Pyocyanin (PYO) | 10 nmol $L^{-1}$ | Electrochemical detection as PYO is redox active | [134] |
| Whatman 3 MM CHR paper | *Pseudomonas aeruginosa* | N/A | Electrochemical detection to determine minimal inhibitory concentration | [136] |

nanomaterials, the design of multilayer device can solve this issue, and nanomaterial fabricated paper devices is attracting more and more attention.

Since μPADs eliminate the need for pre-training by specialists and enables rapid onsite detection, it is more than suitable to be prompted to commercialisation for extensive uses. Data can therefore be collected in largescale, and if location information is enabled e.g., via the integration with smartphone-based sensing technology where global positioning system (GPS) is built-in, the analytes abundance can be mapped and eventually a local, national, and global pathogen profile can be built. Besides, with the advancement of computational sciences including deep learning and IoT, algorithms could be trained and tested with data collected. Fundamental models can be built from the algorithms to improve the precision of the sensor for robust test results, while models can be further developed to reflect current pathogen distribution profiles as well as to predict the trend of pathogen development and transmission with precision. Emerging risks of potential pandemic outbreaks can be evaluated, and therefore timely and effective intervention could be performed to alleviate risks, providing a promising tool to guide decision making. It has been demonstrated that there is the potential of integrating computational sciences for real-time data analysis to monitor reaction dynamics. Sun et al. [137] proposed a method combining paper microfluidics, portable optoelectronic system with deep learning for SARS-Cov-2 detection. Real-time data was fed into neural networks for early prediction analysis, with accuracy, sensitivity and specificity of the prediction reaching 98.1%, 97.6%, and 98.6%, respectively. In addition to nucleic acid, Duan et al. [138] proposed a deep learning-assisted smartphone platform for ultra-accurate testing of paper-based microfluidic colorimetric enzyme-linked immunosorbent assay (c-ELISA) for protein biomarker detection. The proposed algorithm enabled elimination of ambient light influence and gave a >97% accuracy in rabbit IgG concentration quantification. The sensing process was fully automated with 'image in, answer out', with a simple and user-friendly smartphone application developed on smartphone. More work is required to further expand on this interdisciplinary area, contributing to facile in-field detection in resource limited settings. Furthermore, increasing interests have been drawn to the integration of wearable technology with paper-based chips to promote personal health protection. It has shown the capability to analyse sweat to monitor human health conditions, e.g., cortisol excretion as an indicator of stress conditions [139] and uric acid as a risk biomarker for diseases such as type 2 diabetes [140]. In the future, the technology may be further implemented for in-field monitoring of pathogen.

In addition to mainstream applications such as clinical diagnostics, environmental monitoring, food safety and drug discovery, μPADs also hold potential to be applied in other areas such as presumptive tests at crime scenes and forensic analysis [141]. For example, Cromatie et al. [142] proposed a multiplexed μPAD that is capable of the simultaneous colorimetric detection of blood, saliva, semen, and urine. Compared to other applications, there is a lack of studies focusing on this area and we believe that μPADs has a broad application for on-site testing, including body fluids, biomarkers such as nucleic acids and proteins, as well as biochemical molecules such as toxins, and therefore with the potential to carry out onsite crime scene analysis.

While μPAD offer various merits in onsite pathogen detection and holds potential for extensive use under various scenarios, there are still concerns that need to be solved. The inherent cellulose structure of μPAD significantly restricts its performance. There is 'coffee ring effect', where solutes of assay such as reagents, analytes and reaction products tend not to uniformly distribute in the test zones but aggregate around the outer edges, impairing the accuracy and precision of detection results. Besides, there are dramatic variations in structure and layout in paper fibres, which will affect the patterning of hydrophobic barriers into paper and the wicking rates of fluids, and eventually affects the consistency of the results. The transport efficiency of assays in μPAD is inherently low due to the porosity of cellulose that will absorb liquids, and the evaporation exacerbates the situation. This issue is especially more concerning when dealing with samples of small amount or low concentrations, leading to false negatives. In addition, current fabrication techniques may not be adequate to create strong hydrophobic barriers, and thus liquids of low surface tension might leak to unexpected chambers, affecting the test results. Reagents such as enzymes stored in μPAD tend to denature rapidly during shipment and storage, which also impacting on detection accuracy and precision. Finally, the LODs of colorimetric paper-based detection is also not as sensitive as in laboratory, limiting its application in drinking water or food where permitted maximum level is extremely low. In addition, there is also a lack of scalable paper device fabrication methods as most current studies are proof-of-concept [143], focusing mainly on resolution and novelty. However, there is a requirement to improve the mass production of reproducible and reliable μPAD, which will promote the real-world application.

Efforts have been made to circumvent the limitations of μPAD. For example, μPAD is designed to employ non-porous capillary channels for transport to improve delivery efficiency and paper-like porous zones for detection with its size and shape optimised to minimise the 'coffee ring effect'. Bioactive papers have also been proposed to improve sensitivity of μPAD, and one example is to fabricate gold nanoparticles onto paper, while other options include combination between assay reagents and sol-gel materials [144]. Progresses in related areas such as 3D printing and CRISPR/Cas technology also benefit the development of μPAD, improving its analytical performances as well as its capability for use in end users under various condition. For the moment, 3D printing technologies supplement and advance the development of μPADs in three general aspects (i) solid support structures (e.g., the fabrication of housings or other accessory structures): for paper microfluidic device components; (ii) channel barrier (hydrophobic) definition in existing porous materials; and (iii) porous channels for capillary flow [145]. Although currently there are still obstacles that need to be solved, e.g., mass production, integration of additive materials to, for example, to embed electronic circuits within polymers, and direct printing on highly porous material for improved reproducibility of μPAD, the future of 3D printing technology still holds great promise, leading to the next generation of μPAD device.

## Author statement

**Wenliang Li**: Conceptualization, Writing - original draft, Writing - review & editing, **Xuanye Ma**: Writing - original draft, Writing - review & editing, **Yang-Chun Yong**, Writing - review & editing, **Guozhen Liu**: Writing - review & editing, **Zhugen Yang**: Conceptualization, Writing - review & editing, Project administration.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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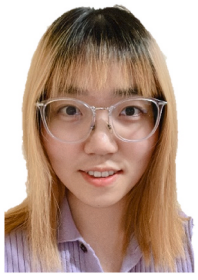

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