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REVIEW

Shaping up field-deployable nucleic acid testing using microfluidic paper-based analytical devices

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Abstract

Rapid, low-cost, and sensitive nucleic acid detection and quantification assays enabled by microfluidic paper-based analytical devices (μPADs) hold great promise for point-of-care disease diagnostics and field-based molecular tests. Through the capillary action in μPAD, flexible manipulation of nucleic acid samples can be achieved without the need for external pumps or power supplies, making it possible to fabricate highly integrated sample-to-answer devices that streamline the nucleic acid extraction, separation, concentration, amplification, and detection. To detect minute amounts of genetic materials from clinical and biological samples, it is also critical to develop sensitive signal readouts that generate physically detectable signals for in-device nucleic acid detection and/or quantification. In this review, we will focus on μPAD approaches for the facile manipulation of nucleic acids and emerging signal transduction strategies allowing sensitive and specific nucleic acid detection in μPAD.

Keywords Paper-based analytical device · Microfluidics · Nucleic acid testing · Point-of-care testing · Field-deployable assays

Introduction

Nucleic acid testing (NAT) that detects genetic markers from clinical or biological samples plays critical roles for disease diagnosis, health surveillance, food safety, and environmental monitoring. By far, NAT is performed predominantly at centralized laboratories, where trace amounts of nucleic acids can be extracted, amplified, and quantified using high-end instrumentation and by well-trained personnel. However, field-based molecular tests, such as molecular diagnosis at or near the site of patient care, known as point-of-care testing (POCT), require NATs that are of rapid response and can be performed at decentralized laboratory settings such as doctor's office or at home. Nucleic acid–based POCT is also critical to address global health challenges faced by many developing countries or remote regions where healthcare resources are very limited and access to central laboratories is impossible. To be used as a POCT in resource-limited settings, an ideal NAT shall meet the "ASSURED" criteria developed by the World Health Organization (WHO), which include *a*ffordable, *s*ensitive, *s*pecific, *u*ser-friendly, *r*apid and robust, *e*quipment-free, and *d*elivered to end users [1]. Towards these ideal criteria, paper with the low-cost and unique capillary action has naturally been recognized as one of the best engineering materials for realizing field-deployable NAT. As such, diverse analytical and microfluidic devices made partially or entirely out of paper, generally known as microfluidic paper-based analytical device (μPAD), have been created and have been widely used as detection platforms for NAT in recent years.

The idea of μPAD was first introduced by Whitesides and coworkers in 2007 [2], opening the possibility to create sophisticated microfluidic systems by simply patterning diverse designs on papers. Comparing to other analytical devices, μPAD offers several unique advantages towards field-based applications. First, paper is a widely available and low-cost material that can be readily manufactured, modified, and patterned. The main component of paper is cellulose which is an abundant polymer existing in plants and chemically stable in weak acids, bases, and commonly used organic solvents. Papers of varying sizes, thicknesses, pore sizes, and chemical

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modifications are widely available, and well-defined channels can be easily created through a diverse range of printing and patterning techniques, such as wax printing, ink-jet printing, screen printing, photolithography, laser direct writing, or even handwriting [3]. Paper can also be physically modified by cutting to form designed shapes. More complicated multi-dimensional paper circuits can also be achieved by simply folding and unfolding the paper with designed channel. Second, because of the hydrophilicity and porosity, μPAD can generate and control liquid flows via capillary action without the need for external pumps. If necessary, external power supplies, such as electricity, can also be used for the manipulation (e.g., electrophoresis) or detection (e.g., electrochemical detection) of nucleic acids in μPAD. Also, due to the high surface area to volume ratio, paper can store the relative high amount of fluids within the fibers than other microfluidic platforms. Thirdly, paper is also an environmentally friendly material that can be easily disposed and degraded. When handling samples containing infectious pathogens or biohazards, it is also possible to minimize the risks of users and potential contamination by simply burning the devices. These advantages have driven the development of diverse μPADs for clinical diagnosis, environmental monitoring, and food safety surveillance. In this review, we will focus on the newly emerged μPADs for NAT with an emphasis on unique designs for in-device manipulation of nucleic acid samples and signal generation/amplification strategies for the nucleic acid detection in μPADs. More discussion on fabrication techniques and other analytical applications of μPADs can be found in several recent reviews [3–5].

Manipulating nucleic acid samples using μPAD

A typical NAT involves three main steps, including nucleic acid sample preparation, amplification, and detection. Towards field-based applications, it is critical to miniaturize each step and eventually integrate all steps into a single portable device. μPAD is an ideal platform to achieve such goals, as it offers simple inexpensive solutions for NAT miniaturization. Moreover, recent realization of multi-dimensional paper machines and origami enables sophisticated sample processing and liquid transportation in μPAD. Highly integrated sample-to-answer μPADs have also been fabricated which streamline all steps of NAT in a single device.

Sample preparation using μPAD

Sample preparation is a critical step in NAT to extract and purify nucleic acids from complicated sample matrix and to remove inhibitors for subsequent nucleic acid amplification. Cellulose paper, such as Whatman FTA card, has already been commercially available for sample collection, nucleic acid storage, and transportation. To further enhance the in-device nucleic acid extraction, purification, and liquid transportation, a variety of μPADs have recently been developed. For example, Fronczek et al. developed a one-dimensional (1D) paper device capable of extracting nucleic acids of *Salmonella typhimurium* from field and clinical samples through filtering and chromatographic interactions between cellulose (or nitrocellulose) with varying components in the sample [6]. The authors found that proteins, lipids, and other cell lysates were retained close to the inlet of their device, whereas nucleic acids migrated further in the paper channel and thus could be separated from the sample matrix. Using this simple μPAD, the nucleic acid extraction can be accomplished within 5 min and readily compatible with nucleic acid amplification such as PCR. However, the use of this device requires a sample lysis and dilution step prior to loading the sample. An ideal μPAD shall allow direct raw sample preparation without the need for prior treatments. To achieve this goal, Govindarajan et al. introduced a 3D paper origami device that integrates the lysis of the *Escherichia coli* bacterial cells and nucleic acid extraction into a single device without using external equipment or treatment (Fig. 1a) [7]. This device was fabricated by stacking layers of Mylar sheets with repositionable adhesive and cellulose paper. All reagents including lysis buffer, extraction buffer, and washing buffers were preloaded and dried at the different layers of the device to achieve the field-ready sample preparation. Upon introducing the raw sample into the central inlet, a series of lysis, washing, and extraction steps could be activated by simply folding the designated layers of the paper origami. The overall extraction process starting from the raw sample can be completed within 1.5 h without the use of external power or within 1 h using a heater block with an extraction limit as low as 33 CFU/mL.

For samples containing only trace amount of nucleic acids, it is ideal to concentrate nucleic acids during the extraction step. One viable strategy is to integrate μPAD with electrokinetic extraction techniques, such as isotachophoresis (ITP) and ion concentration polarization (ICP) [8, 9, 13]. Using ITP, nucleic acids can be separated and concentrated from complex sample matrix using electric field and a discontinuous buffer system created by a leading electrolyte (LE) and a trailing electrolyte (TE). In 2015, Li et al. reported a 3D multi-layered paper-based ITP device capable of concentrating DNA samples [8]. As shown in Fig. 1b, the device consists of wax-patterned filter paper with multiple 2-mm-diameter circular paper wells that were concertina folded into a 2-mm-thick channel and were held in the middle of two reservoirs containing the LE and TE buffers, respectively. Due to the short channel length, a high electric field of ~ 16 KV/m can be generated for ITP using two 9-V batteries. Using this device, over 100-fold enrichment was achieved for DNA having lengths of up to 1517 bps within 10 min. When integrating

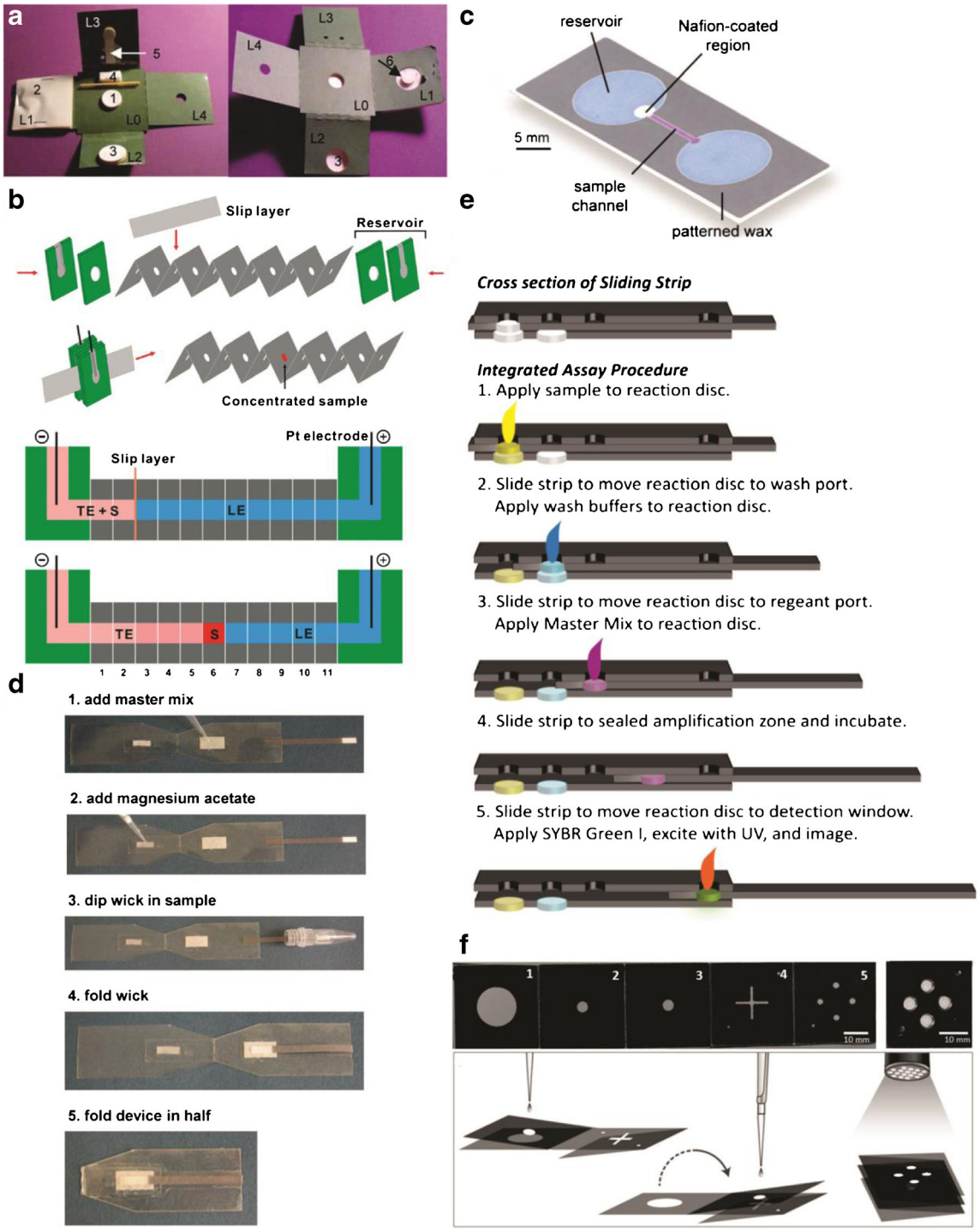

Fig. 1 **a** Front (left) and back (right) side view of the 3D μPAD [7] (Copyright 2012, The Royal Society of Chemistry). **b** Multi-layer paper-based ITP platform for nucleic acid concentration [8] (Copyright 2015, The Royal Society of Chemistry). **c** ICP-μPAD device for DNA preconcentration, separation, and detection [9] (Copyright 2015, American Chemical Society). **d** Schematic illustration of nucleic acid amplification by RPA on paper and plastic device [10] (Copyright 2012, The Royal Society of Chemistry). **e** 3D "paper-machine" that integrates DNA extraction, amplification, and detection [11] (Copyright 2015, American Chemical Society). **f** 3D μPAD for detecting nucleic acids from whole blood samples [12] (Copyright 2016, Wiley)

with ICP, μPAD can not only concentrate DNA but also separate DNA based on their sizes or separate double-stranded DNA (dsDNA) from single-stranded DNA (ssDNA). Recently, Gong et al. developed such an ICP-μPAD capable of simultaneously preconcentrating, separating, and detecting DNA fragments in clinical samples [9]. The device was

fabricated by patterning wax barriers on nitrocellulose paper, which defines two reservoirs connected by a sample channel (Fig. 1c). To enable ICP, one reservoir was partially coated with cation-selective nanoporous Nafion. Upon loading the sample at the sample channel, electrical field was applied across the channel and all anionic species including DNA samples were focused and separated based on their charges because of the dedicate balance between electrophoretic migrations at the ion depletion zone at the interface of the nanoporous Nafion and the electroosmotic flow. Using this device, as low as 150 copies/mL of HBV DNA fragments in human serum samples were successfully preconcentrated, separated, and detected within 10 min, allowing for early diagnosis of hepatitis B without the need for any nucleic acid amplification step.

In-device nucleic acid amplification

To enable the sensitive and specific detection of specific DNA or RNA sequences, nucleic acid amplification is commonly required. Therefore, an ideal μPAD for field-applicable NAT shall also provide in-device nucleic acid amplification capacity. As standard PCR requires thermal cycles, in-device PCR was found to be an enormous challenge and to date was unsuccessful. On the other hand, significant progress has been made to integrate μPAD with isothermal nucleic acid amplification techniques. The in-device amplification was first reported by Rohrman et al. in 2012, where HIV DNA was successfully amplified using recombinase polymerase amplification (RPA) in a paper-tape hybrid origami device (Fig. 1d) [10]. Similar to the 3D paper-origami for sample preparation, the in-device RPA was also achieved by storing key assay reagents (master mix and magnesium acetate) into different layers and then activating the reaction by simply folding the device. A long sample wick strip with wax-patterned hydrophobic arm was also created to facilitate the direct sample introduction from the microcentrifuge tube and thus eliminated the need for pipetting. By using a lateral flow assay (LFA) as readout, the authors were able to detect as low as 10 copies of HIV DNA. Liu et al. reported a paper-based device capable of carrying out target-induced rolling circle amplification (RCA) to produce massive quantities of DNA amplicons for the subsequent detection [14]. This paper device was fabricated by patterning 96-microzone on a nitrocellulose paper with each test zone of 4 mm diameter. The authors then printed RCA reagents onto the paper device within a pullulan sugar film, allowing the maintenance of more than 90% activity in 4 °C after 15 days. It was also observed that RCA was more proficient on paper than in solution, likely due to the higher local concentration of immobilized DNA, enabling a detection limit of single digit picomolar level. In addition to RPA and RCA, other isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP), strand displacement amplification (SDA), and helicase-dependent amplification (HDA) have also been successfully integrated into μPAD to achieve the in-device amplification of the target nucleic acids [15–20].

NAT using highly integrated sample-to-answer μPADs

Fully integrated μPADs that streamline the sample preparation, nucleic acid amplification, and detection are highly desirable to fulfill the final goal of field-deployable NAT at low-resource environment [21]. One of the first fully integrated μPADs was introduced by Whitesides and coworkers in 2015, where they developed a 3D "paper machine" that fully integrated sample preparation, LAMP, and detection in a low-cost, single-use format [11]. As shown in Fig. 1e, this device was designed to contain three major layers of magnetic sliding strips that could be slid to control the serial introduction of sample, wash buffer, amplification reagents, and detection reagents, meanwhile also dynamically sealed to prevent evaporation during incubation steps. The middle lay of the device contains a reaction disk fabricated using Whatman FTA paper. The whole operation procedure only requires the sequential sliding of the middle layer through each port on the top and bottom layers followed by adding sample, washing buffer, LAMP Master Mix, and incubation in 65 °C for 1 h. Using this "paper machine," the authors were able to detect as low as 1 copy of the synthetic dsDNA representing the *malB* gene fragment of *E. coli* and consistently detect 5 *E. coli* cells in human plasma.

To further push μPAD for direct NAT in human blood sample, Xu et al. introduced a 3D origami paper device capable of whole blood nucleic acid extraction, separation, LAMP, and multiplexed nucleic acid analyses in a single device (Fig. 1f) [12]. The device was fabricated by patterning wax onto multiple foldable layers of filter paper. A glass fiber disk was also inserted to the middle layer for nucleic acid extraction. The NAT was initiated by loading the human blood sample onto the glass fiber disk and followed by folding the device to enable cell lysis and DNA extraction on the glass-fiber paper. The purified DNA was then transferred to the amplification panel by another folding step and split to four independent paper wells where species-specific LAMP reagents were deposited. The system was sealed using an acetate film to prevent evaporation, and amplification was carried out at 63 °C for ~45 min before the naked-eye detection with a handheld UV lamp. Using this device, simultaneous detection of multiple pathogens including *Plasmodium falciparum* (98%), *P. malariae* (96%), and *P. vivax* (98%) from either finger-prick fresh blood sample or frozen blood was achieved within a single paper device. The NAT in whole blood sample can also be achieved using electrokinetic paper-based devices that enable nucleic acid separation and preconcentration in a single step. For example, Bender et al. recently described an

electrokinetic paper-based device that integrated ITP with RPA [22]. This device consisted of a glass fiber strip that connected with two electrolyte reservoirs. A plasma separation membrane was placed on the top of the strip, so that when loading the whole blood samples, only plasm could wick onto the glass fiber strip. By loaded the TE buffer and LE buffer with RPA reagent into two reservoirs and applied electric field onto strip, as low as 10^4 copies/mL of DNA was extracted, concentrated, and amplified from human blood sample by ITP-RCA in a single step.

As nucleic acid amplification requires incubation at elevated temperatures, most abovementioned devices still require external reusable equipment such as incubator, water bath, and heater, which not only adds the cost and complexity of the NAT, but also increases the risk of cross-contamination. To address this challenge, Tang et al. recently developed a fully disposable and integrated μPAD for nucleic acid extraction, amplification (HDA), and detection using LFA [23]. Moreover, the authors further equipped their device with a sponge-based reservoir for extraction, an integrated battery, a positive temperature coefficient ultrathin heater, temperature control switch, and on-device dried reagents for HDA. Using this fully integrated device, the authors successfully detected *Salmonella typhimurium* in samples including wastewater, milk, juice, and egg.

Despite the simplicity offered by the μPAD for liquid handling and sample processing, the detection of target nucleic acids or amplicons remains an analytical challenge. Comparing to standard solution-based assays, nucleic acid detection using μPADs is generally of lower analytical performance (e.g., sensitivity) due to the paper composition and potential sample losses during the liquid transportation. As such, it is critical to develop novel signal transduction strategies with improved sensitivity and specificity but without compromising the simplicity and cost offered by μPADs. In the next section, we will focus our discussion on the recent advances and new trends for nucleic acid detection in μPADs.

Signal transduction strategies for nucleic acid detection in μPAD

Strategies that translate nucleic acids or amplicons into physically detectable signals in paper are critical and sometime the bottleneck step to ensure the sensitive and specific NAT using μPAD. The detection of specific nucleic acid sequences can be achieved by either direct capturing through DNA hybridization or through amplification by using sequence-specific primers. Colorimetric readout using lateral flow assays (LFA) and fluorescent readout using DNA-intercalating dyes are two most commonly used signal transduction approaches in μPAD [24–31]. Advanced nucleic acid sensing strategies making use of CRISPR/Cas systems [32–34], DNA hybridization probes [35–47], DNA nanotechnology [48–51], and synthetic biological approaches [52–54] have also been introduced to μPAD, representing a new trend for designing better paper-based NAT for field-based applications.

Lateral flow assays

LFAs, such as pregnancy tests, are one of the earliest paper-based immunoassays for POC diagnosis. LFAs can be readily used for NAT by labeling the target nucleic acids with affinity ligands or antigens such as biotin and digoxigenin. The labeling can be achieved through PCR or isothermal amplification, where a set of two primers are labeled with two distinct ligands. In the presence of the target DNA or RNA, the double-stranded amplicon bearing both ligands can be captured at the testing line of LFA meanwhile recruiting a signal reporter such as gold nanoparticle or fluorescent dye for subsequent colorimetric or fluorescence readout [24–26]. One challenge in this type of LFA assays is the potential false positive caused by the nonspecific amplification and primer dimers. To address this challenge, Phillips et al. recently introduced a tagged strand displacement probe to LAMP (Fig. 2a) [24]. This probe was designed to bind to the targeted loop region of the LAMP products by toehold-mediated strand exchange, a reaction that is highly sequence specific due to the thermodynamic penalties of the initiating branch migration. As such, comparing to a direct labeling of LAMP primers, this probe eliminated false positive test bands in LFA. Using this strategy, the authors were successfully detected as few as 3.5 *Vibrio cholera* and 2750 *E. coli* bacteria without any false positive or false negative interpretation.

It is also possible to modify the lateral flow strip with complementary DNA probes capable of capturing single-stranded amplicons through DNA hybridization (Fig. 2b) [27]. As lateral flow strip offers sufficient spatial resolution to position multiple DNA probes, multiplex nucleic acid detection can be achieved on a single LFA device. In addition to serving as a stand-alone paper-based device for NAT, LFAs have also been adapted to other μPADs to create fully integrated sample-to-answer devices [21, 23]. Because of the simplicity, LFA is also an attractive low-cost engineering platform that translates emerging ultrasensitive and specific NAT into field-deployable diagnostic assays. One exciting example is the recent paper-based SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) assay for the detection of Zika and Dengue viruses and gene mutations in clinical samples [32–34]. SHERLOCK is a novel nucleic acid detection platform recently developed by Zhang and coworkers, which combines isothermal preamplification with Cas13 to detect single molecules of RNA or DNA [32]. The same research group has recently introduced the second version of SHERLOCK (SHERLOCKv2), where multiplexed detection

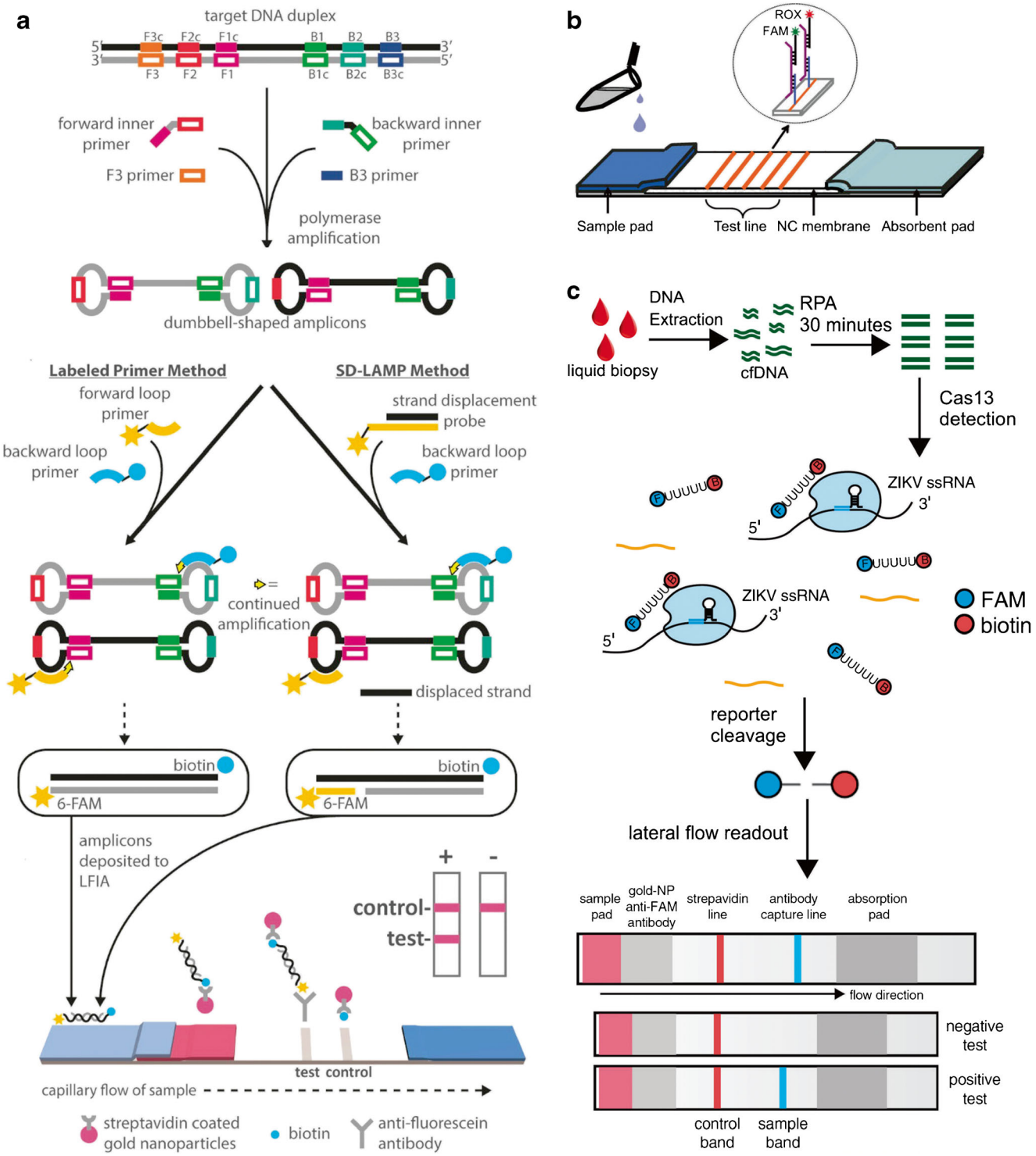

Fig. 2 **a** Strategies for the detection of LAMP products using LFA [24] (Copyright 2018, American Chemical Society). **b** Multiplexed detection of nucleic acids by capturing multiple DNA amplicons on a single lateral flow strip through hybridization [27] (Copyright 2014, American Chemical Society). **c** Schematic illustration of a typical work flow of SHERLOCKv2 assay with LFA as a paper-based readout [33] (Copyright 2018, American Association for the Advancement of Science)

could be achieved by using four orthogonal CRISPR enzymes (LwaCas13a, CcaCas13b, PsmCas13b, and Cas12a) that preferentially cleave certain dinucleotide combinations when CRISPER RNA binds to its target [33]. By further engineering the cleavage substrates, commercially available LFAs can be used as readout for the SHERLOCKv2 assay. As shown in Fig. 2c, virus RNAs were first extracted from the clinical sample and then preamplified using RPA to accumulate the

targets that trigger the collateral activities of Cas proteins through the binding to the CRISPR RNA. The substrate was designed to contain biotin at one end and FAM at the other end. As the lateral flow strip contained a streptavidin-modified control line and a protein A–modified test line, abundant reporters accumulated anti-FAM antibody-gold nanoparticle conjugates at the control line. In the presence of the target nucleic acids, the cleavage of the reporter using the CRISPR-Cas machine reduced accumulation at the control line and results in signal on the test line. The paper-based SHERLOCK assay allowed the instrument-free detection of ZIKV or DENV ssRNA at 2 aM detection limit within 90 min [34]. Moreover, this paper-based detection system is also sensitive to single nucleotide mutations, which has been demonstrated by the detection of mutations in liquid biopsies of nonsmall cell lung cancer patients [33].

Intercalating dyes

Despite the wide application to NAT, LFAs are generally qualitative and requiring additional modifications to the primers. To further push the quantification capacity and simplify the assay protocol, fluorogenic DNA-intercalating dyes have been widely used in μPAD. Fluorogenic DNA-intercalating dyes are the workhorses in biochemical laboratories for nucleic acid staining and quantification. Because of the strong intramolecular quenching, these dyes possess no or very low fluorescence in solution [28, 29]. However, the binding of such dyes to nucleic acids or amplicons limits the self-quenching and thus turns on the fluorescence. As no labeling or washing steps are required, it is not surprising that intercalating dyes are one of the most widely used signal readout strategies in μPAD when nucleic acid amplification is involved. However, to facilitate the in-device visual detection of nucleic acids, an UV lamp or light box is required. To eliminate the need for external light sources, Roy et al. explored the visual, colorimetric detection of LAMP amplicons in μPAD using a chromogenic DNA intercalator, crystal violet (CV) [30]. CV is violet in color, however can be converted into leucocrystal violet (LCV) in the presence of sodium sulfite. The binding of CV to dsDNA can effectively prevent this color transition and change the colorless LCV to the violet CV, allowing the colorimetric sensing of the genes of *Sus scrofa* (porcine) and *Bacillus subtilis* in paper. To fulfill the quantification capacity, subsequent data extraction and analysis steps are required using imaging software (e.g., ImageJ) after collecting the data using a digital camera. One possible solution to simplify the quantification process for end users is to use smartphone camera and to develop software capable of automated data processing. It is also possible to achieve the direct in-device nucleic acid quantification by using digital readout that can be counted without the need for external readers. For example, our group recently developed a paper-based quantitative DNA reader (qPDR) capable of translating the conventional fluorescence-based reading into the measurement of distance as readout (Fig. 3a) [31]. The idea of qPDR was established on an interesting phenomenon that cellulose paper can effectively retain and turning on the fluorescence of a commonly used DNA-intercalating dye, SYBR Green I (SG-I). The binding strength was found to be in between dsDNA and ssDNA. Therefore, dsDNA such as PCR amplicons could effectively elute SG-I from the sample loading zone to the testing zone, whereas ssDNA such as primers has no elution effect. As the migration distance of SG-I in the test zone is quantitatively determined by the concentrations of the dsDNA, quantitative information can be readily obtained by visually examining the migration distance. By integrating qPDR with a portable thermal cycler, we were able quantify as low as 10 copies gene fragments of *Trichuris trichiura* (TT) worms that were expelled from school age children at the rural area of Honduras. One drawback of using DNA-intercalating dye is that these dyes bind ubiquitously to dsDNA or ssDNA and thus do not provide the specificity for discriminating single nucleotide mutations. μPADs making use of intercalating dyes may also lead to false positive results if nonspecific nucleic amplification occurs. To further enhance the assay accuracy and specificity, many sequence-specific DNA hybridization probes have been developed and integrated to μPADs.

Hybridization probes

Short synthetic DNA probes that are complementary to the target ssDNA or RNA are one of the most powerful approaches for the sequence-specific detection of nucleic acids. When combining with μPAD, a typical design involves the modification of the paper substrate with a capture DNA probe and the labeling of the target with a detection DNA probe [35–45]. The sandwiched binding complex can then be detected through the detection DNA probe that is modified by an enzyme, a fluorescent dye, or a nanoparticle [35–38]. More advanced optical or electrochemical detection platforms have also been introduced to μPAD by further modifying the capture probes within the paper substrates with luminescent nanomaterials or microelectrodes or engineering the detection probes with advanced DNA nanotechnology approaches [40–49]. For example, Krull and coworkers have developed a series of paper-based solid-phase nucleic acid hybridization assays by chemically immobilizing quantum dots (QDs) as donors to enable fluorescence resonance energy transfer (FRET) assays (Fig. 3b) [39, 40]. Comparing to conventional uses of QDs as passive fluorescent labels, the paper-based solid-phase FRET assays show several advantages, including the rapid hybridization kinetics (< 2 min), low detection limit (~ 300 fmol), and high multiplexity. The same group also explored the immobilization of upconversion nanoparticles

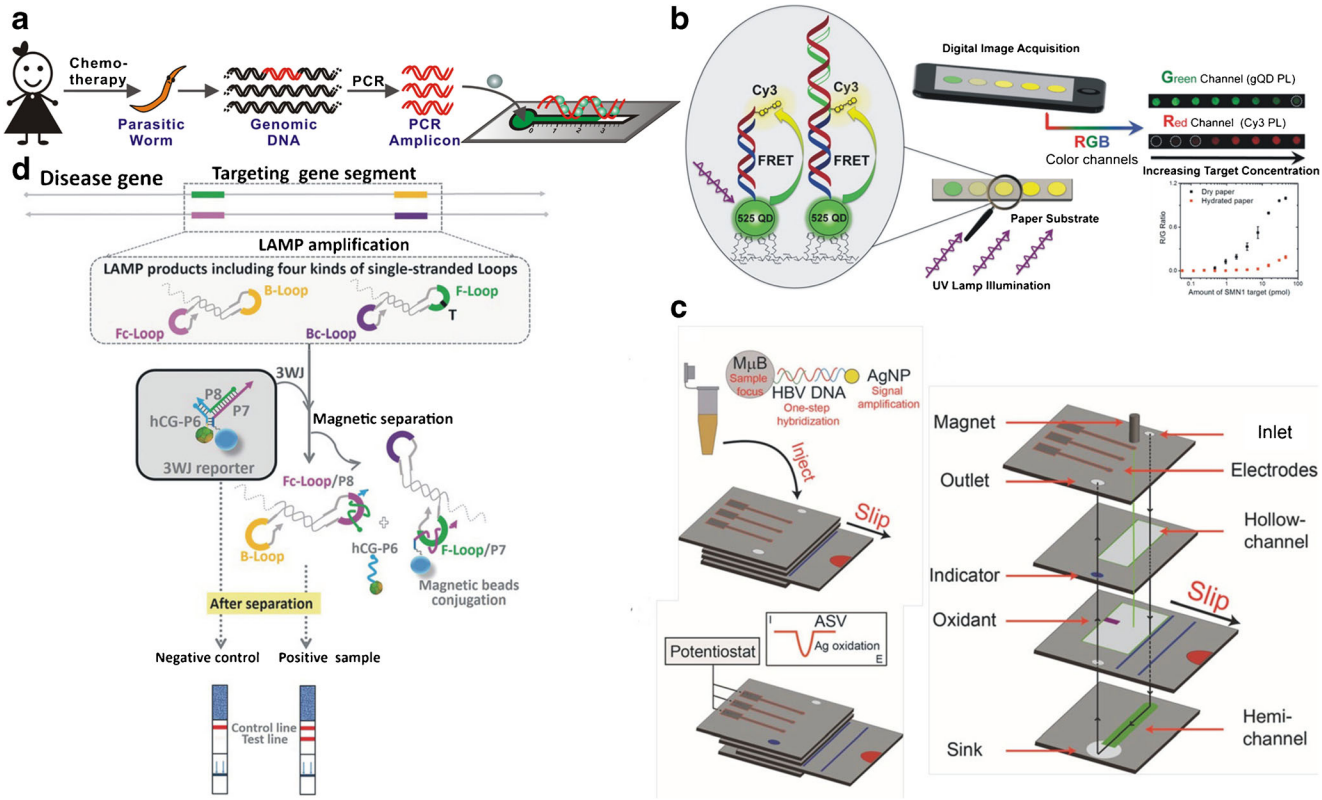

Fig. 3 **a** Distance-based reader-free nucleic acid quantification using paper-based quantitative DNA reader [31] (Copyright 2018, American Chemical Society). **b** FRET-based DNA detection using quantum dot–modified paper device [39] (Copyright 2014, American Chemical Society). **c** Electrochemical detection of HBV DNA using 3D oslip-DNA [45] (Copyright 2015, American Chemical Society). **d** Method for the detection of LAMP product using a pregnancy test strip [46] (Copyright 2017, Wiley)

(UCNPs) as energy donors that allowed the luminescence resonance energy transfer (LRET) on paper, where QDs could be used as sensitive fluorescence readout [41, 42]. The paper-based FRET assay is also readily compatible with isothermal DNA amplification [40]. When integrating with HDA, the authors have successfully detected as low as zeptomoles of target nucleic acids.

Electrochemical readout is also an attractive readout for paper-based NAT, as microelectrodes can be easily printed on paper and the whole device can be miniaturized for field applications [43–45]. To achieve this goal, synthetic DNA probes modified with electrochemical labels have been used for the detection of specific sequences in μPADs. For example, Li et al. described a 3D paper-based electrochemical sensor called oslip-DNA (o stands for origami) for the detection of HBV [45]. As shown in Fig. 3c, the device was fabricated by first patterning channels using wax printing on chromatographic paper and then removing the paper using razor blade to create hollow channels. The three electrodes were then added by stencil printing. To operate the oslip-DNA, one-step sample incubation was used to capture DNA-functionalized silver nanoparticles (AgNPs) onto DNA-functionalized magnetic microparticles (MμBs) through the target DNA. After washing the unbound AgNPs, the mixture was loaded to the inlet of the oslip-DNA and MμBs carrying AgNPs were captured directly at the working electrode by using a small rare-earth magnet. Once capturing the MμBs, the slip layer was pulled to a functional position, which resulted in the release of a strong oxidant ($KMnO_4$). $KMnO_4$ rapidly oxidized AgNPs and released large amounts of Ag^+ ions. The Ag^+ was then electrochemically deposited onto the working electrode and quantified using anodic stripping voltammetry (ASV). As the overall cost was estimated to be 0.36 USD and the detection of HBV-specific nucleic acids could be done within 5 min, this device holds good potential for field-based NAT. The detection limit of this device was 85 pM, suggesting a nucleic amplification step might be necessary to push the detection limit to the clinically relevant levels.

Besides designing and crafting new μPADs, it is also possible to engineer standard pregnancy test strips as readout for NAT using the concept and strategies in dynamic DNA nanotechnology. Du et al. recently introduced this idea, where human chorionic gonadotropin (hCG) was used as a label for DNA hybridization probes (Fig. 3d) [46]. The hCG-DNA probe was then designed to hybridize to LAMP products through a toehold-mediated strand displacement. When loaded onto the pregnancy test strip, the resulting hCG-LAMP complexes were too large to migrate and thus generated an

"off" signal. An alternative "turn-on" assay was also designed, where hCG-DNA was released from a bulky three-way junction reporter through a toehold-mediated strand displacement with the LAMP products. Using this assay, as few as 20 copies of Ebola virus templates could be detected in both human serum and saliva using a commercially available pregnancy test strip. Moreover, this assay could also be adapted to distinguish a common melanoma-associated SNP allele from the wild-type sequence. In addition to the toehold-mediated strand displacement reactions [46, 47], more advanced concept and strategies of the emerging DNA nanotechnology such as catalytic hairpin assemblies and hybridization chain reactions have also been integrated with μPADs as isothermal and enzyme-free signal amplifiers to enhance the analytical performance of paper-based NATs [48, 49].

Synthetic biology approaches

Recent advances in the field of synthetic biology have yield several powerful synthetic gene networks capable of sensing the surrounding environment and generating a measurable output [50–52]. One intriguing question is that can such powerful synthetic biological system be integrated into μPAD to create low-cost field-deployable NAT for diagnosis at resource-limited environments? To achieve this goal, Collins and coworkers have recently developed two impactful synthetic biological approaches, including toehold switches [50] and paper-based synthetic gene network [51]. Toehold switches are a class of de novo–designed prokaryotic riboregulators that activate gene expression in response to cognate RNAs with arbitrary sequences (Fig. 4a). This system is composed of two RNA strands: the switch and the trigger. The switch RNA contains the coding sequence of the gene that is regulated by an upstream hairpin-based processing module containing both a strong ribosome binding site (RBS) and a start codon. The trigger RNA (the target) can hybridize to the hairpin through a toehold-mediated strand displacement reaction and expose the RBS and start codon, thereby initiating translation of the gene of interest. The system was designed such that the trigger RNA did not possess complementary bases to the RBS or the start codon and thus could be generalized as a sensor for any target nucleic acids. To further enable the use of toehold, the same group also developed the paper-based synthetic gene network that was achieved by freeze drying cell-free biological components into paper to create materials with the fundamental transcription and translation properties of a cell (Fig. 4b). Remarkably, these cell-like papers are stable at room temperature for over 1 year and can be activated by simply adding water. The on-paper colorimetric or fluorescent readout can be readily achieved by embedding mRNAs for expression chromogenic enzymes such as LacZ or fluorescent proteins. By further integrating the paper-based protein expression system with mRNA sensors operated by toehold switches, the authors have successfully developed a panel of 24 sensors that could distinguish between the Sudan and Zaire strains of the Ebola virus (Fig. 4c). The same system has also been applied to the in-field diagnosis of Zika virus with clinically relevant sensitivity [52]. To do so, an isothermal RNA amplification technique known as NASBA (nucleic acid sequence–based amplification) was used to amplify and accumulate the trigger RNAs for the subsequent toehold switch reactions on paper (Fig. 4d). In a typical workflow, Zika virus RNAs were directly collected from serum or saliva samples and amplified using NASBA. The reaction mixture was then loaded onto the paper disk to rehydrate the reagents for the expression of the enzyme LacZ capable of converting the yellow chlorophenol red-b-D-galactopyranoside into the purple chlorophenol red on paper. The authors also combined NASBA with a CRISPR/Cas9 module that selectively cleaves the target DNA sequence possessing a PAM domain, which allowed the discrimination between American and African ZIKV.

Conclusions and perspective

Miniaturization of nucleic acid testing into decentralized field-deployable assays has long been a focus of analytical and bioanalytical science community. Since first introduced in 2007, microfluidic paper-based analytical devices have already made significant impact to the realization of the field-deployable NAT. μPADs capable of nucleic acid extraction, purification, separation, and concentration have been developed which greatly simplify and accelerate sample preparation procedures for NAT. Many in-device amplification and detection modules have also been introduced and thus made it possible to fabricate highly integrated sample-to-answer devices. To further push the analytical performance of μPADs, research efforts have also been made to establish novel strategies and mechanisms to generate better visual, optical, or electrochemical readouts. Novel designs have been made to the classic lateral flow assays, intercalating dyes, or DNA hybridization probes to enable new detection paradigms for μPADs. Concept and strategies in the emerging dynamic DNA nanotechnology, CRISPR Cas systems, and synthetic biology have also been introduced and open new opportunities for designing μPAD with better sensitivity, specificity, and accuracy. To facilitate the comparison of varying μPAD designs for sample processing and/or signal readout, we summarize representative strategies in terms of sample preparation, nucleic acid amplification, and detection in Table 1. While significant progress has been made, challenges and new trends are emerging. First, fully integrated μPADs with better quantification capacity will be highly desirable for real-world applications. By far, most integrated μPADs rely on commercially available LFAs, which provide limited sensitivity and

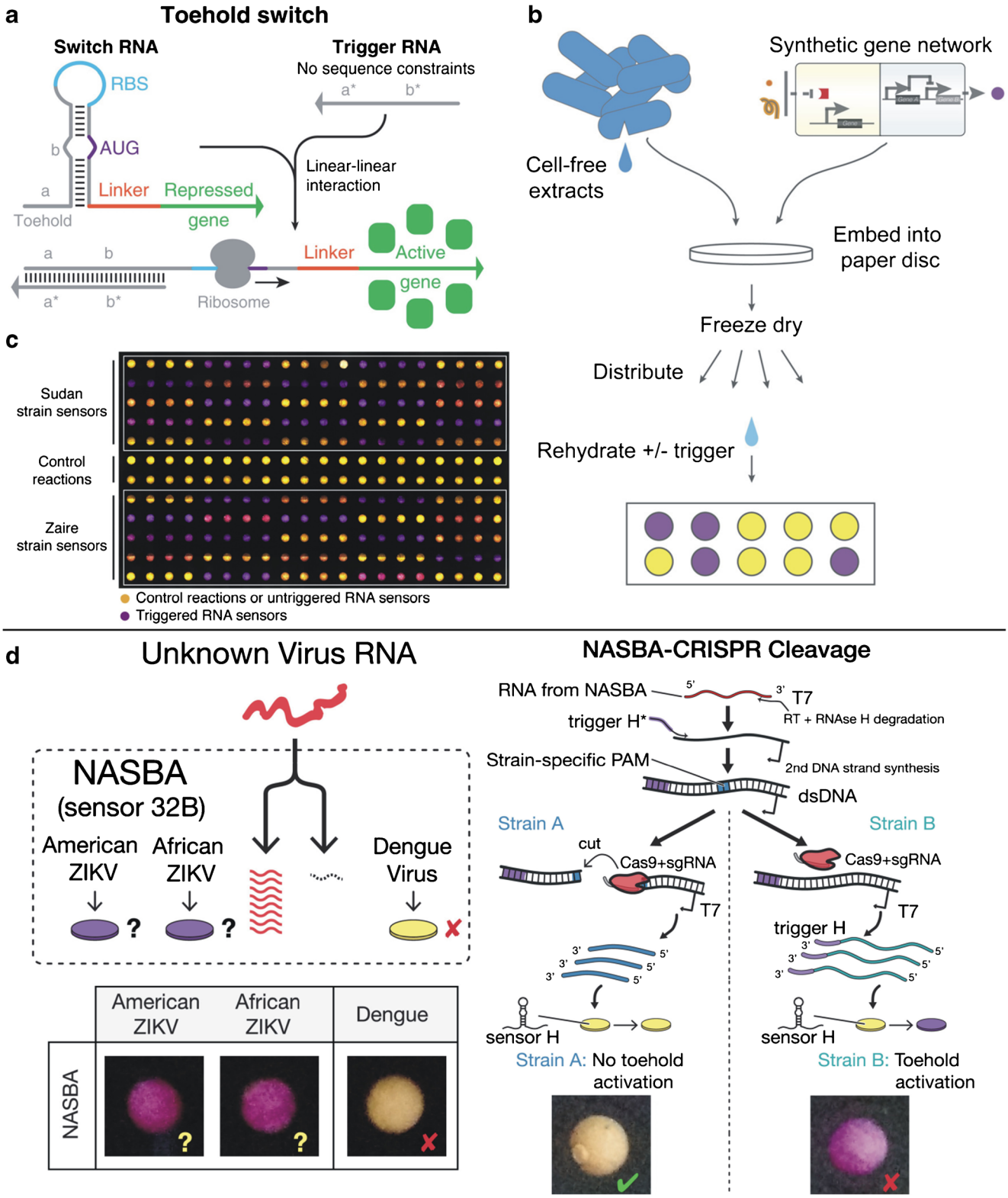

Fig. 4 **a** Schematic illustration of the concept of toehold switches [50] (Copyright 2014, ELSEVIER). **b** Preparation and activation of paper-based synthetic gene network [51]. **c** Diagnosis of Ebola viruses using a panel of paper-based synthetic biologic sensors [51] (Copyright 2014, ELSEVIER). **d** In-field diagnosis of Zika virus and stain discrimination using NASBA and NASBA-CRISPER cleavage assays [52] (Copyright 2016, ELSEVIER)

Table 1 Summary of representative μPAD designs and applications

Ref.	Sample		μPAD	Sample preparation			In-device amplification	Detection		
	Target	Matrix		Pretreatment	Extraction	Preconcentration		Method	LOD	Operation time
Fronczek et al. (ref. [6]	*Salmonella typhimurium*	Whole blood and fecal sample	Cellulose and nitrocellulose paper	Sample dilution	Chemical lysis	×	×	Fluorescence	10^3 to 10^4 CFU/mL	5 min
Govindarajan et al. (ref. [7]	*E. coli*	Pig mucin (simulating sputum)	Millipore cellulose paper and Fusion 5 membrane	×	Chemical lysis	×	×	PCR and gel electrophoresis	33 CFU/mL	1.5 h without external power/1 h with heater block
Li et al. (ref. [8]	Synthetic DNA	Purified DNA	Whatman grade 1 cellulose paper	×	×	ITP	×	Fluorescence	> 100-fold enrichment	~ 10 min
Gong et al. (ref. [9]	HBV	Human semen	Nitrocellulose paper	Sample dilution and cell lysis	×	ICP	×	Fluorescence	150 copies/mL	~ 10 min
Rohrman et al. (ref. [10]	HIV	Purified DNA	Glass fiber, cellulose paper, and Fusion 5 membrane	Sample dilution and labelled with FAM and biotin	×	×	RPA	LFA	10 copies of DNA in 10 μL	15 min
Liu et al. (ref. [14]	Hepatitis C virus/ microRNA from human breast cancer cell line	Purified DNA and microRNA	Nitrocellulose membrane	×	×	×	RCA	Radioactive tracer, colorimetric, and fluorescence	10 pM	×
Connelly et al. (ref. [11]	*E. coli*	Human plasma	Whatman FTA paper and Ahlstrom 226 paper	×	Whatman FTA paper	×	LAMP	Fluorescence	1 copy of DNA /5 cells per 10-μL sample	×
Xu et al. (ref. [12]	*Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium* pan	Whole blood	Glass fiber and cellulose paper	×	Chemical lysis	×	LAMP	Fluorescence	5 parasites /μL	45 min
Bender et al. (ref. [22]	HIV-1	Whole blood	Glass fiber	×	Plasma separation membrane	ITP	RPA	Fluorescence	10^4 copies/mL	> 20 min
Tang et al. (ref. [23]	*Salmonella typhimurium*	Wastewater, milk, juice, and egg	Cellulose paper and Fusion 5 paper disk	×	Chemical lysis and alkaline extraction	×	HDA	LFA	10^2 CFU/mL (wastewater and egg), 10^3 CFU/mL (milk and juice)	~ 1 h
Phillips et al. (ref. [24]	*E. coli* and *Vibrio cholera*	Whole blood, human plasma, and pond water	LFA strip	Cell lysis and primer modification and LAMP amplification	×	×	×	LFA	3.5 *Vibrio cholera* cells and 2750 *E. coli* bacteria	×
Zhang et al. (ref. [33, 34]	ZIKV and DENV	Whole blood, serum, and saliva	LFA strip	Lyse viral particles, inactivate ribonucleases, and RPA	×	×	×	LFA	1 copy/μL	< 2 h

Table 1 (continued)

Ref.	Sample		μPAD	Sample preparation			In-device amplification	Detection		
	Target	Matrix		Pretreatment	Extraction	Preconcentration		Method	LOD	Operation time
Roy et al. (ref. [30]	DNA of porcine and *Bacillus subtilis*	Purified DNA	Cellulose paper	LAMP amplification	×	×	×	Colorimetric	1 pg/μL for porcine and 10 pg/μL for *Bacillus* bacteria	65 min
Wang et al. (ref. [31]	*Trichuris trichiura*	Clinical worm samples	Whatman grade 1 cellulose paper	Genomic DNA extraction and PCR amplification	×	×	×	Distance	10 copies of DNA in 10 μL	~10 min
Noor et al. (ref. [40]	DNA of survival of motor neuron 1	Purified DNA	Chromatography paper	Thermophilic HDA preamplification	×	×	×	Fluorescence	37 zmol in 6 mL	~3 h
Li et al. (ref. [45]	DNA of HBV	Purified DNA	Chromatographic paper	Incubation of target DNA with labelled AgNPs and MμBs	×	×	×	Electrochemical	85 pM	Less than 5 min
Du et al. (ref. [46]	Plasmid DNA of Zaire ebolavirus and BRAF	Human serum and saliva	Commercial pregnancy test strips	LAMP amplification and primer modification	×	×	×	LFA	20 copies of Ebola templates per sample	~2.5 h
Pardee et al. (ref. [51, 52]	RNA of Ebola virus and Zika virus	Purified RNA	Cellulose paper	RNA extraction	×	×	In-device gene expression	Colorimetric and fluorescence	30 nM of trigger RNA	~3 h

quantification capacity. Devices making use of chromogenic or fluorogenic dyes often require additional readers and tedious data processing to extract the quantitative data. With advanced optical add-on and automated imaging processing software, smartphone can serve effectively as a user-friendly detection platform. It is also possible to fabricate μPAD with reader-free digital readout, such as distance [31], time [53], or colored segments [54] that can be counted by the end user. Second, it is also essential to design μPADs for challenged clinical and biological samples. For example, the diagnosis and management of parasitic diseases at the developing world require nucleic acid extraction from worm eggs in stool and soil samples. So far, most μPADs were designed and verified using liquid biopsies such as serum, saliva, or sputum, and effective nucleic acid extraction and subsequent detection represent an analytical challenge for future research. Thirdly, most of the novel signal readout strategies making use of nanomaterials, DNA nanotechnology, and synthetic biology approaches have not been integrated with the in-device sample preparation and amplification modules yet. Fully integrated NAT devices that leverage the multi-dimensional μPAD for sample preparation and newly emerged paper-based readout systems will be highly desirable for field-ready applications. Towards these challenges, we anticipate that the unique properties of μPADs will be continuously explored, generating powerful analytical tools for in-field molecular testing and point-of-care diagnosis in the near future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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