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REVIEW ARTICLE

Current Advances in Paper-Based Biosensor Technologies for Rapid COVID-19 Diagnosis

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Abstract
The global coronavirus disease 2019 (COVID-19) pandemic has had significant economic and social impacts on billions of people worldwide since severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in Wuhan, China, in November 2019. Although polymerase chain reaction (PCR)-based technology serves as a robust test to detect SARS-CoV-2 in patients with COVID-19, there is a high demand for cost-effective, rapid, comfortable, and accurate point-of-care diagnostic tests in medical facilities. This review introduces the SARS-CoV-2 viral structure and diagnostic biomarkers derived from viral components. A comprehensive introduction of a paper-based diagnostic platform, including detection mechanisms for various target biomarkers and a COVID-19 commercial kit is presented. Intrinsic limitations related to the poor performance of currently developed paper-based devices and unresolved issues are discussed. Furthermore, we provide insight into novel paper-based diagnostic platforms integrated with advanced technologies such as nanotechnology, aptamers, surface-enhanced Raman spectroscopy (SERS), and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas. Finally, we discuss the prospects for the development of highly sensitive, accurate, cost-effective, and easy-to-use point-of-care COVID-19 diagnostic methods.

Keywords COVID-19 · SARS-CoV-2 · Paper-based biosensors · Lateral flow assay (LFA) · Point-of-care testing (POCT)

1 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in Wuhan, China, in November 2019, rapidly spreading worldwide and causing a global pandemic of coronavirus disease 2019 (COVID-19). This led to more than 500 million confirmed cases and more than 6.2 million deaths in more than 215 countries, as of April 28, 2022 [1]. The COVID-19 outbreak has had a significant economic and social impact on billions of people worldwide. Strong government sanctions, such as social distancing, quarantine, and lockdowns, have paralyzed businesses, severely weakening the global economy. This has caused many people to lose their jobs or has severely affected their household economy. Moreover, as the COVID-19 outbreak continues for an extended period, the medical community and staff, as well as the general public, are facing significant stressful situations (e.g., anxiety and depression) due to the intractable virus. Although effective vaccines and therapeutics for COVID-19 have been already developed and widely disseminated, transmission is still rapid and sustained owing to the emergence of the highly contagious variants of SARS-CoV-2 caused by mutations [2, 3].

Since the beginning of the COVID-19 pandemic, SARS-CoV-2 has accumulated mutations, and several types of SARS-CoV-2 variants identified by viral genome sequencing have been discovered in various global regions. Recent studies have shown that SARS-CoV-2 variants are more transmissible than the wild-type virus [4–7]. Additionally, the SARS-CoV-2 variants can bypass immune protection against exposure to the same virus conferred by previous vaccinations and infections [8, 9]. Fortunately, variant viruses do not continuously evolve to increase the risk of disease severity. The Omicron variant, which is currently dominant worldwide, is less severe than previous strains, especially compared to the Delta variant [10]. However, there is no guarantee that the subsequent dominant variants will not cause severe disease symptoms. In the future,

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devastating SARS-CoV-2 variants may emerge with higher viral transmissibility, disease severity, and vaccine-bypassing efficacy through the continued accumulation of mutations in the virus genome. To date, only a limited number of therapeutics, such as remdesivir and dexamethasone, have shown some effectiveness in reducing the mortality or severity of COVID-19. However, universally applicable therapeutics for asymptomatic and mild to moderate COVID-19 patients have not yet been developed. [11]. In the absence of universal antiviral therapeutics and reduced vaccine efficacy due to the immune escape of the variants, accurate, fast, and efficient diagnostic tools to identify patients with COVID-19 during the early stages of infection are vital for the control and further prevention of this disease.

Timely diagnosis, effective treatment, and future prevention are the most critical factors for successfully managing COVID-19 [12]. Among these, timely diagnosis plays an essential role in preventing and slowing the spread of the disease as the first line of defense. Early diagnosis of infected individuals enables immediate isolation of patients with highly contagious viruses (e.g., SARS-CoV-2), effectively controlling the spread of the disease. Moreover, early diagnosis allows physicians to provide immediate treatment, increasing the chances of cure and survival [13]. From the first outbreak of COVID-19 until now, the gold standard for confirming COVID-19 infection has been quantitative reverse transcription PCR (RT-qPCR) which amplifies small amounts of viral RNA in samples collected from an infected individual. However, this standard technique requires the analysis to be performed by trained experts and in a fully equipped laboratory, which inevitably increases the cost and time of testing. In a public health emergency where COVID-19 cases are increasing and COVID-19 testing capacity needs to be expanded, the introduction of more inexpensive, faster, easier-to-use diagnostic testing that analyzes samples at the point of use and screens for COVID-19 in a larger population is essential [14]. To compensate for the shortcomings of the current RT-qPCR assays, paper-based rapid diagnostic tests, especially lateral flow assays (LFA), have recently received widespread attention as an alternative to suppress the rapid spread and reinfection of COVID-19 [15]. Numerous LFA-based rapid diagnostic devices for diagnosing COVID-19 have been developed with great success, especially in resource-constrained environments and rapidly growing numbers of patients [16, 17]. So far, LFA-based rapid diagnostic technology has been used as an adjunct to RT-qPCR for COVID-19 confirmation due to its limited performance in terms of assay sensitivity and reproducibility [15]. However, extensive and intensive efforts are being made in academia and industry to improve the performance of LFA-based rapid inspection.

This review introduces the SARS-CoV-2 viral structure and diagnostic biomarkers derived from viral components. Then, a comprehensive introduction of paper-based diagnostic platforms, including device components, detection mechanisms for different target markers (e.g., nucleic acid, antigen, and antibody makers), and COVID-19 commercial kits, is presented. The intrinsic limitations related to the poor performance of the currently developed paper-based devices are also discussed. Furthermore, novel paper-based diagnostic platforms integrated with advanced technologies such as nanotechnology, aptamers, surface-enhanced Raman spectroscopy (SERS), and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas are presented. These novel detection methods are promising for improving the diagnostic performance of previously reported paper-based tests. Finally, we discuss the prospects for the development of highly sensitive, accurate, cost-effective, and easy-to-use point-of-care COVID-19 diagnostic methods.

2 SARS-CoV-2 Viral Structure and Diagnostic Targets

The SARS-CoV-2 belongs to the genus β-coronavirus and comprises a single positive-strand RNA with a genome of ~ 30 kb. The SARS-CoV-2 genome codes ten genes that produce 26 proteins [18]. The genes are arranged in the sequence 5′ cap structure-replicase (open reading frame1/ab, ORF1/ab)-structural proteins with a [spike (S)-envelope (E)-membrane (M)-nucleocapsid (N)]-3′ poly (A) tail [19]. Among these genes, unique and specific nucleotide sequences representing SARS-CoV-2, such as genomic fingerprints, are targets for COVID-19 diagnosis. The genome sequence of SARS-CoV-2 was shared through the Global Initiative on Sharing All Influenza Data (GISAID) platform, on January 12, 2020 [20]. Subsequently, various primer and probe sets have been developed to amplify specific viral RNA sequences, and the WHO has posted these primer–probe sets [20–23], enabling the rapid development of COVID-19 nucleic acid amplification tests (NAATs). More than 100 RT-qPCR kits have been designed and prototyped and are the United States food and drug administration (FDA) emergency use authorization (EUA)-approved for COVID-19 diagnosis. These kits aim to amplify specific regions of viral genes, such as structural protein genes (N, E, S, and M) and confirmation genes (ORF1ab and RNA-dependent RNA polymerase (RdRp)) [20, 23, 24] (Fig. 1a). RT-qPCR offers high accuracy and very low analytical sensitivity; thus, it has been used as the gold standard for confirming COVID-19 infection.

The SARS-CoV-2 genome encodes four major structural and functional proteins: the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins (Fig. 1b) [11, 25, 26]. The S protein comprises two functional subunits, S1 and S2; S1 acts as a key protein for selective binding with the

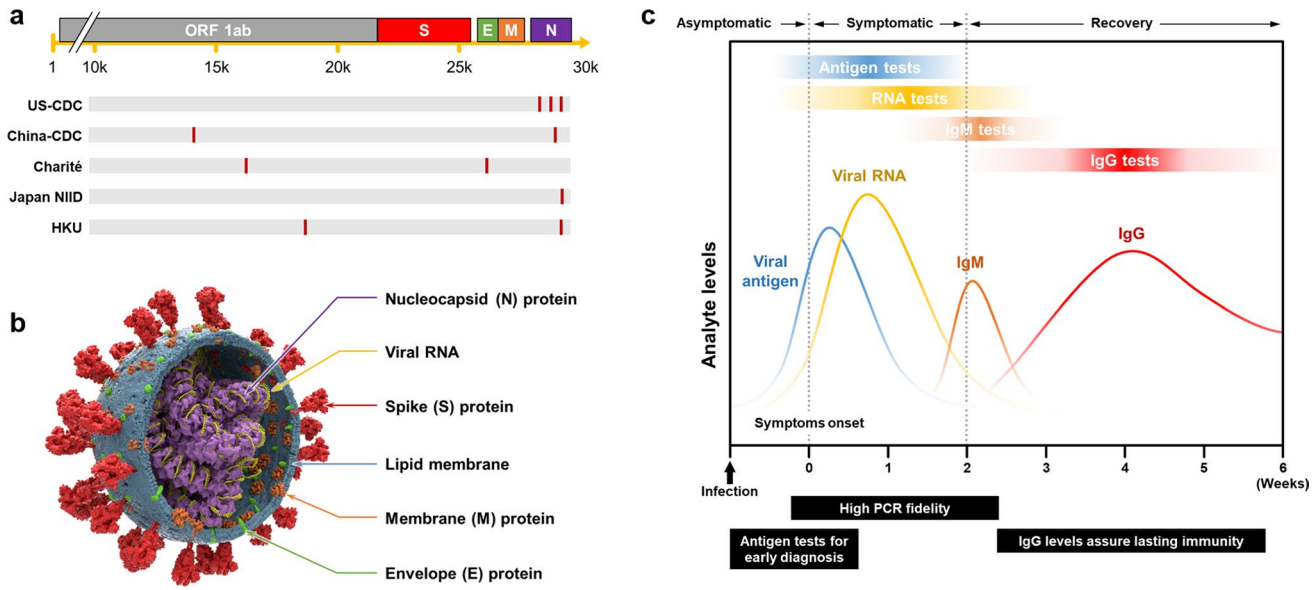

Fig. 1 SARS-CoV-2 genome, structure comprising major proteins, and variation levels of biomarkers across the duration of the infection. **a** The SARS-CoV-2 genome codes ten genes, and the genes are arranged in the sequence 5′ cap structure-ORF1/ab-spike (S)-envelope (E)-membrane (M)-nucleocapsid (N)-3′ poly (A) tail. Primer–probe sets for SARS-CoV-2 RNA amplification developed by research groups around the world [US Centers for Disease Control and Prevention (US-CDC) – target sequences: 28,287 ~ 28,358, 28,681 ~ 28,752, 29,164 ~ 29,230; China CDC – target sequences: 13,342 ~ 13,460, 28,881 ~ 28,979; Charité–Universitätsmedizin Berlin in Germany (Charité) – target sequences: 15,431 ~ 15,530, 26,269 ~ 26,381; National Institute of Infectious Disease in Japan (Japan NIID) – target sequence: 29,125 ~ 29,282; and University of Hong Kong (HKU) – target sequences: 18,778 ~ 18,909, 29,145 ~ 29,254]. **b** SARS-CoV-2 is mainly composed of four major proteins: spike (S) (red), membrane (M) (orange), envelope (E) (green), and nucleocapsid (N) (purple) proteins. **c** Temporal dynamics of the viral load and antigen and antibody levels. Since the types and amounts of biomarkers present in a patient's body fluid differ depending on the stage of infection, it is critical to select an appropriate biomarker and a method that can effectively detect it for an accurate diagnosis of COVID-19

host cell receptor, angiotensin-converting enzyme 2 (ACE2), while S2 mediates membrane fusion [11, 27, 28]. The N protein is the most abundant protein in the virus and plays a crucial role in packaging and protecting viral genomic RNA. The N protein is highly immunogenic and rarely changes with disease progression [29–32]. The M protein is the most abundant structural protein that defines the shape of a virus, while the E protein is the smallest major structural protein involved in virus assembly and pathogenesis [28]. The S and N proteins are considered the most valuable antigenic biomarkers for diagnosing COVID-19 in the various detection methods for SARS-CoV-2 [33, 34].

In contrast, specific antibodies produced by the immune response to protect the body from SARS-CoV-2 infection could be another option for diagnosing COVID-19. Immunoglobulin M (IgM) is the first line of defense during viral infections appearing in the blood after SARS-CoV-2 infection, and increases rapidly, which is an indicator of early stage infection. IgG antibodies subsequently produced by the IgM antibodies are responsible for immunological memory and long-term immunity, which serves as the body's immune defense system to avoid reinfection with the same pathogen [35, 36]. IgA antibodies limit the entry of microorganisms and antigens into the susceptible mucosal barrier through respiratory mucosal epithelial protection and homeostasis regulation [37–39]. IgA antibody responses appear early and are characterized by intense and sustained maintenance [40]. Therefore, the detection of IgA antibodies is advantageous in the early infection stages.

Assay sensitivity is affected by the temporal profile of the viral load (or concentration of the biomarkers) across the duration of the infection. This is a critical factor in determining the diagnostic accuracy. Several studies have suggested the temporal dynamics of the viral load, antigen and antibody levels after SARS-CoV-2 infection [41–44]. In general, the viral load increased rapidly from the time of infection, peaked, and then decreased rapidly (within a few days) (Fig. 1c). After 10 days of infection, the viral load is reduced by a factor of 100 or more [41, 45]. Considering the virus structure, the quantities of viral RNA and antigens were consistent with the trend of the viral load. Therefore, the optimal time for detecting viral RNA and antigens is approximately 7 days, immediately after symptom onset. Conversely, viral RNA cannot be detected in the early or late phase of infection. Another study analyzed the serological response of COVID-19 patients to viral infections [42]. Approximately 10 days after symptom onset, the IgM response to SARS-CoV-2 is predominant over other immunoglobulins. After

approximately 15 days, the IgM response decreased and disappeared. IgG antibodies are produced later than IgM production, 10–14 days after symptom onset; however, IgG antibodies persist and are detectable for a long time [43, 44]. IgM antibodies can mainly be used for the early detection of SARS-CoV-2 infection, whereas IgG antibodies may be more appropriately used to identify past infections.

The type and amount of biomarkers present in the patient's body fluid differed depending on the infection stage of the COVID-19 patient (Fig. 1c). To analyze these biomarkers, an appropriate sample preparation process according to the specimen and analysis method is also essential. In general, the upper respiratory tract sample is used in the case of RT-qPCR and antigen tests, and if possible, the lower respiratory tract sample is inspected simultaneously. Generally, the nasopharyngeal swab method is more sensitive than the nasal swab method for specimen collection in COVID-19 [46]. However, the nasopharyngeal swab method should be performed by a trained healthcare provider. The Centers for Disease Control and Prevention (CDC) noted that if both nasopharyngeal and oropharyngeal specimens can be collected, the sensitivity of the test can be maximized by combining the two specimens in one tube. On the other hand, the nasal swab method is more comfortable than the nasopharyngeal swab method and can be used easily by the general public; therefore, many diagnostic kits have been developed for nasal swab samples. Before the RT-qPCR test, RNA extraction from the sample is a crucial step to getting accurate amplification results [47, 48]. It should be carried out using proven equipment and reagents, and contamination should be prevented throughout the sampling and analysis process. In the case of the antibody tests, a small amount of blood should be collected through venipuncture or the fingerstick method. To reduce interference or increase sensitivity, any clots or erythrocyte sediments in the samples may be removed after the appropriate clotting or centrifuge method. It is essential for serum or plasma analysis kits, but these processes may be omitted for several whole blood or fingerstick kits [49, 50]. Therefore, for accurate diagnosis of COVID-19, it is crucial to select an appropriate biomarker and a method that can effectively detect these biomarkers according to the stage of infection.

3 Paper-Based Diagnostic Platform for COVID-19 Diagnosis

While the economically developed world has access to many advanced medical tools, trained personnel, and resources to perform diagnostic tests for maintaining health, these similar resources are unfortunately not as accessible in developing countries. Rapid and accurate diagnosis is the first step toward improving healthcare conditions in developing countries. In dire situations, such as the COVID-19 pandemic, the damage is inevitably more severe for countries marginalized from good medical services. In these countries, the supply of the COVID-19 vaccine is delayed and accurate diagnostic techniques for early diagnosis are extremely limited. Many disease-related deaths, including COVID-19, would have been preventable if the disease had been diagnosed earlier and followed by appropriate treatment. Moreover, large-scale diagnostic testing is critical to contain the COVID-19 epidemic, even in developed countries with well-established healthcare services [51]. Standard diagnostic techniques (i.e., RT-qPCR) are limited in the rapid screening of confirmed cases from a rapidly elevated number of suspected cases. For these outbreaks, it is crucial to have medical diagnostic platforms that can analyze samples on-site and provide immediate results [13].

Paper-based analytical devices show great potential in delivering POC diagnostic systems to the developing world because of their remarkable properties such as biocompatibility, porosity, ease of modification, flexibility, chemical inertness, eco-friendliness, and ease of storage and transportation [52, 53]. Moreover, various sample types can be applied to paper-based analysis equipment, and sample transfer is possible without requiring additional power owing to capillary force. Over the last three decades, paper-based POC tests have been developed for various biomedical applications and launched as both 'over-the-counter' products, such as glucose monitoring and pregnancy testing, and 'professional market' products that can diagnose infectious disease, cardiac markers, diabetes, lipidoses, hemopathies, and several cancers.

Paper-based POC tests have also played a crucial role in the current COVID-19 pandemic. Many researchers in academia and industry have made intensive efforts to develop a simple, convenient, fast, sensitive, and accurate technology that can detect SARS-CoV-2. The technology that can best meet the strong demand for practical POC diagnostic tests in COVID-19 control is paper-based POC testing. Paper-based POC diagnostic platforms range from simple one-dimensional platforms, such as dipstick and LFA, to complex three-dimensional platforms, such as microfluidic paper-based assay devices (μPAD) and electrochemical paper-based assay devices (ePAD). Among these, LFA is a highly mature paper-based diagnostic technology that researchers and manufacturers have invested the most effort and cost in developing COVID-19 diagnostic kits. A typical LFA comprises a sample pad, a conjugate pad, a nitrocellulose membrane, and an absorbent pad. The sample flow begins on the sample pad and meets the signal molecules that have dried on the conjugate pad. All biomarkers, antigens, antibodies, and SARS-CoV-2 RNA used to detect SARS-CoV-2 can be applied to LFA.

3.1 LFA-Based Diagnostic Platform for Detecting Viral Antigens

Unlike RT-qPCR, antigen-based diagnostics directly detect the presence of SARS-CoV-2 and related proteins in a sample taken from a nasopharyngeal swab or nasal passage without sample pretreatment and amplification. Therefore, it can provide a diagnosis of COVID-19 with faster and easier results at lower cost than RT-qPCR. Antigen-based diagnosis is based on immunoassay reactions that involve antigens and antibodies. The configuration of the general LFA diagnostic platforms for detecting SARS-CoV-2-specific antigens is shown in Fig. 2a. All proteins constituting SARS-CoV-2 can be targeted to diagnose COVID-19, but antigen tests for COVID-19 have been developed mainly targeting the S and N proteins [54–56]. To detect the SARS-CoV-2 antigens, a specific antibody pair that recognizes different regions of the target antigen is required. A capture antibody is immobilized on a nitrocellulose membrane to form a test line (first line), and another antibody is labeled with a signal molecule, mainly gold nanoparticles, and serves as the detection antibody. Additionally, the control line (second line) also serves to check whether the sample flowed through the nitrocellulose membrane, and additional Ig-types of antibodies that could not affect the test are used. When a nasopharyngeal swab sample of a patient with COVID-19 is loaded into the LFA device, the sample containing the target antigens flows along with the LFA strip by capillary force and first encounters the detection antibody. Target antigens are captured by both detection and capture antibodies to form a sandwich complex. After 15–20 min of sample loading, the appearance of color in the test and control lines is confirmed visually or by a portable analyzer.

Although various paper-based antigen diagnostic tests have been developed, the sensitivity of the rapid antigen test is unclear and is lower than that of RT-qPCR. The limit

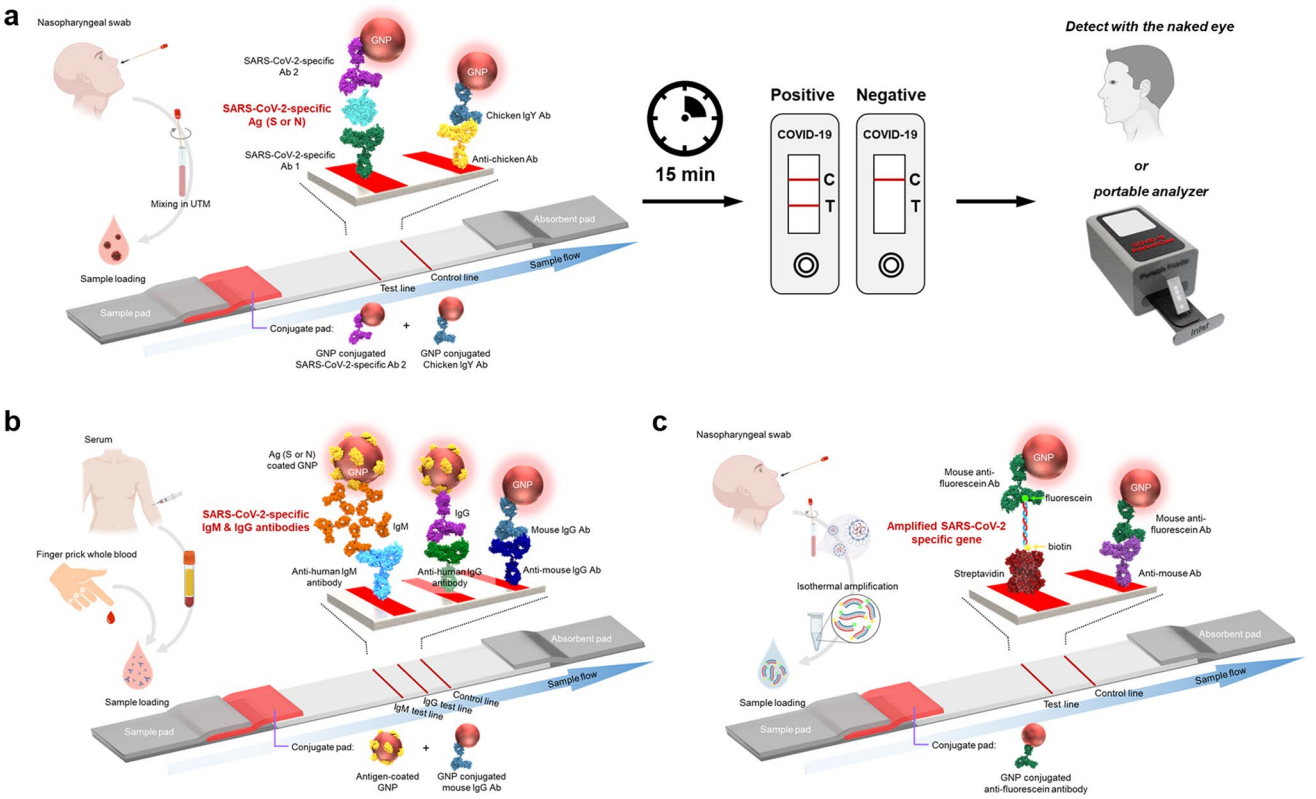

Fig. 2 Paper-based diagnostic platforms including device components, detection mechanisms for different target markers, **a** antigens, **b** antibodies, and **c** RNA. **a** To detect the SARS-CoV-2 antigens, a specific antibody pair is required. These capture and detection antibodies detect SARS-CoV-2-specific antigens (S and N proteins) while forming a sandwich complex. After 15–20 min of sample loading, the appearance of color in the test and control lines is confirmed visually or by a portable analyzer. **b** In serological tests (detecting IgM and IgG antibodies), the N (or S) proteins of SARS-CoV-2 are conjugated with gold nanoparticles and used as signal molecules to detect IgM and IgG antibodies. Anti-human IgM (or IgG) antibodies are immobilized on a nitrocellulose membrane to form test lines. When the sample contains the SARS-CoV-2-specific IgM or IgG antibodies, the antibodies are bound to the N (or S) protein-conjugated gold nanoparticles and finally bound to the test line, resulting in vivid color. **c** Isothermal amplification techniques combined with an LFA contribute to achieving POC tests for SARS-CoV-2 RNA detection. First, an isothermal amplification process is performed for target RNA amplification, and then an LFA reaction is performed so that the results can be easily checked

of detection (LOD) of antigen tests is approximately 10^5 copies/mL, while that of RT-qPCR is as low as 10^2 copies/mL [57, 58]. False-negative results may occur when the concentration of the target antigen in the clinical specimen is below the analytical sensitivity of antigen tests. Several studies have been conducted to overcome these limitations. Liu et al. presented a novel nanozyme-based chemiluminescence paper assay for detecting SARS-CoV-2 S antigen. In this case, nanozyme (Co-Fe@hemin-peroxidase) and chemiluminescent immunoassays were integrated with LFA to achieve sensitivity (360 $TCID_{50}$/mL) comparable to that of an ELISA method [59]. To improve the performance of the previous signal molecule, gold nanoparticles [60], latex beads [61], cellulose nanobeads [62], and fluorescent microparticles [30] have been introduced into LFA. These signaling molecules have higher signal intensities, resulting in an approximately tenfold improvement in sensitivity compared with previous gold nanoparticle-based LFAs.

Our group also proposed a novel rapid detection method for the SARS-CoV-2 S antigen. Using the cellular receptor for SARS-CoV-2, angiotensin-converting enzyme 2 (ACE2), the SARS-CoV-2 S1 antigen was successfully detected in clinical specimens of COVID-19 patients (Fig. 3a) [63, 64]. Furthermore, we developed SARS-CoV-2 N antigen-specific single-chain variable fragment crystallizable fragment (scFv-Fc) fusion antibodies using phage display technology and applied them to the LFA platform (Fig. 3b) [62]. This scFv-Fc-based rapid diagnostic test showed high specificity that could distinguish even the N protein of SARS-CoV. Baker et al. developed a glyconanoparticle consisting of multivalent gold nanoparticles bearing sialic acid derivatives [65]. They discovered that the N-acetyl neuraminic acid is bound to the S protein and developed LFAs that exploit this interaction as a detection mechanism. This glycoprotein-based LFA showed high selectivity for the SARS-CoV S protein.

3.2 LFA-Based Diagnostic Platform for Detecting Virus-Specific Antibodies

LFA allows for qualitative detection of antibodies in blood samples. When the human body is infected with SARS-CoV-2, the immune system is triggered to fight the virus. Several immunoglobulins, including IgA, IgG, and IgM, are produced during this immune response, inactivating the virus and protecting the body from further infection [66–68]. Serological testing for detecting SARS-CoV-2-specific antibodies has many distinctive advantages: 1) identification of current and past infection, 2) wider detection window, 3) more uniform distribution of antibodies in the blood (increased reproducibility), 4) higher stability and less susceptibility to degradation, and 5) enabling the discovery of short-term and long-term antibody responses.

The configuration of the serological LFA platforms for detecting IgM and IgG antibodies is shown in Fig. 2b. There are some differences in the placement and composition of reagents for serological tests compared to antigen-based LFA. The N (or S) proteins of SARS-CoV-2 are conjugated with gold nanoparticles and used as signal molecules to detect IgM and IgG antibodies. In serological LFA, anti-human IgM (or IgG) antibodies are immobilized on a nitrocellulose membrane to form test lines (T1, T2), and a control line is formed using secondary antibodies produced in other hosts, such as rabbits and mice. When the sample containing the SARS-CoV-2 specific IgM or IgG antibodies is introduced to the LFA strip, the antibodies bound to the N (or S) protein-conjugated gold nanoparticles and flow together, finally binding to the test line, resulting in vivid color.

IgM antibodies first appear in the serum a few days after infection and become detectable approximately 5–10 days after symptom onset. IgM antibodies are present in serum for several weeks, followed by IgG production. Thus, IgM antibodies can be used as an indicator of early stage infection and IgG antibodies can be used as indicators of current or prior infection [12]. Even though gold nanoparticle-based IgM antibody detection tests have been developed [69], further improvement is required owing to their low sensitivity, false-negative rates, and inaccuracy. Zeng et al. developed a lateral flow method combined with an IgG-IgM immunochromatographic assay. Compared with a single IgG (61.76%) and IgM (82.35%) tests, the combined IgG-IgM (85.29%) strip had higher sensitivity [70]. Efforts to improve the performance of lateral flow immunochromatographic assay (LFIA) for simultaneous detection of SARS-CoV-2-specific antibodies have increased [71–73]. Research to enhance analytical sensitivity has also been conducted. Peng et al. introduced a photon-counting approach to quantify the LFA while improving sensitivity (Fig. 3c). They quantified and measured the density of SARS-CoV-2 antibodies using simple laser optical analysis [74]. Additionally, Roda et al. developed a dual optical/chemiluminescence format for LFA for affordable and ultrasensitive detection sensitivity (Fig. 3d) [75]. The optical signals were measured with a simple smartphone camera-based device, and ultrasensitive chemiluminescence signals were obtained with a contact imaging portable device based on cooled CCD cameras. This IgA-LFA diagnostic test can be useful for non-invasive monitoring of the initial immune response to COVID-19.

3.3 LFA-Based Diagnostic Platform for Detecting Viral RNA

Although RT-qPCR is the gold standard possessing high sensitivity, specificity, and accuracy, it is labor-intensive and relies on expensive instrumentation. Moreover, this technique is time-consuming and requires professional expertise;

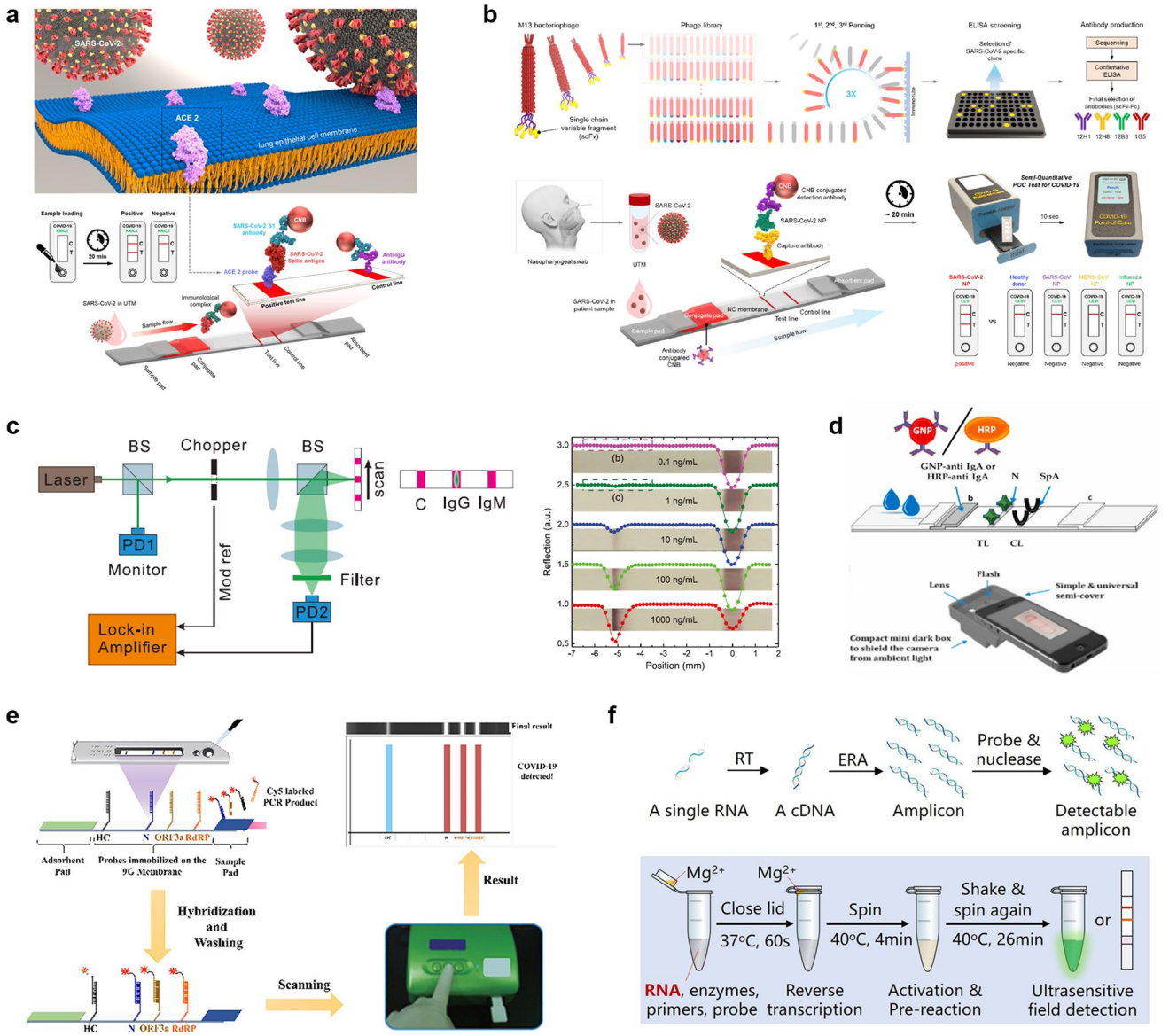

Fig. 3 **a** Cellular receptor (ACE2)-based LFA for detecting SARS-CoV-2 S1 antigen, reproduced with permission from [63], copyright 2021 Elsevier. **b** Development of scFv-Fc-based LFA for detection of the SARS-CoV-2 N protein. Highly sensitive and specific scFv-Fc fusion proteins are rapidly screened by phage display technology, reproduced with permission from [62], copyright 2021 Elsevier. **c** Configuration of detecting system to quantify LFA results with the photon-counting approach and representative results for IgG antibody detection (concentrations range: from 1000 to 0.1 ng/mL), reproduced with permission from [74], copyright 2020 AIP. **d** LFA strip to detect anti-SARS-CoV-2 IgA antibody and the simple and universal smartphone reader to detect the optical signal from LFA, reproduced with permission from [75], copyright 2021 Elsevier. **e** Lateral Flow Strip Membranes (LFSM)-based on highly specific and sensitive detection of SARS-CoV-2. The LFSM assay allows simultaneous detection of the multiple regions of SARS-CoV-2 RNA in a sing test, reproduced with permission from [77, 76], copyright 2020 ACS. **f** Principle of reverse transcription-enzymatic recombinase amplification (RT-ERA). The RT-ERA has the capability of ultrasensitive, field-deployable, and simultaneous dual-gene detection of SARS-CoV-2 RNA, reproduced with permission from [79], copyright 2020 Springer nature

thus, it has limited utility for POC testing. As an alternative to traditional RT-qPCR, a novel LFA was developed for the simultaneous detection of SARS-CoV-2 genes, RdRp, ORF3a, and N genes [76]. The PCR product was obtained by RT-PCR, followed by an LFA assay for 30 min at 25 °C, in which the detection limit was ten copies/test for each gene (Fig. 3e). However, the total assay time still takes approximately 2 h (including 100 min for the PCR reaction).

Isothermal nucleic acid amplification is an alternative strategy that allows amplification at a constant temperature and eliminates the need for heavy equipment, such as thermocyclers [12]. This technique is characterized by high

sensitivity, specificity, convenience, and low cost. Therefore, integrating this technique into an LFA contributes to achieving POC tests for SARS-CoV-2 RNA detection (Fig. 2c). Zhu et al. developed multiplex reverse transcription loop-mediated isothermal amplification (mRT-LAMP) coupled with LFA for diagnosing COVID-19 [77]. By targeting ORF1ab and N gene sequences, isothermal amplification was performed in a single step, and the simultaneously amplified genes were easily interpreted as LFA to achieve COVID-19-specific RNA diagnosis. The platforms showed a detection limit of 12 copies/reaction with no cross-reactivity. The total analysis time for the diagnostic test is 1 h. A qualitative test was developed based on recombinase polymerase amplification (RPA) coupled with LFA [78]. This assay targeted the N gene of SARS-CoV-2 and detected as low as 0.25–2.5 copies/μL of SARS-CoV-2 N gene-containing plasmid. The assay consists of a two-step reaction, an RPA reaction, and an LFA readout; however, the assay requires an additional cDNA synthesis step. Xia et al. introduced an ultrasensitive (single-copy level) field-deployable approach to detect the SARS-CoV-2 gene by applying reverse transcription-enzymatic recombinase amplification (RT-ERA) (Fig. 3f) [79]. ERA is an isothermal amplification method that is a modified version of RPA. By pairing the Exo probe for N and S genes, the two genes were simultaneously detected in one reaction with an extremely low detection limit (one copy level). For POC test applicability, the authors used an affinity probe to detect RNA in the LFA system, and the visual LOD of this approach was as low as one ag (10^{-18} g) of the N gene.

3.4 Microfluidic and Electrochemical Paper-Based Analytical Devices (μPADs & ePADs) for Diagnosing COVID-19

Typical LFAs are cost-effective, fast, and comfortable for COVID-19 testing. However, the sensitivity and specificity are still controversial [80]. It also limits clinical practice as it cannot offer quantitative results for analytes [81]. Although LFA-based diagnostic tests have steadily improved their performance during the COVID-19 pandemic and have achieved remarkable achievements in rapid self-testing for COVID-19, they have a limited ability to perform only unidirectional fluid manipulations. The flow manipulation (direction, flow rate, and flow volume) enables multiplex and multistep assays, providing quantitative information and improving detection sensitivity [82–84]. Microfluidic paper-based analytical devices (μPADs) have been an attractive diagnostic platform that combines the advantages of paper-based biosensors with the capabilities of conventional microfluidic devices. Using simple patterning methods, such as photolithography, inkjet etching, wax printing, screen printing, and PDMS printing, hydrophilic channels/hydrophobic barriers can be easily created, and the ability to manipulate flow is conferred to the μPADs [83]. Unlike conventional LFAs, the flow rate can be directed in multiple directions (horizontal and vertical) depending on the type and design of the μPAD, allowing for the quantitative detection of multiple analytes in a single device. In addition, the μPADs can contribute the improving sensitivity and specificity by controlling the incubation time of the analytes and adding specific steps, such as the washing step. Furthermore, compared with traditional microfluidic devices, μPADs have inherent advantages such as low-cost, simple fabrication, no external power source, and good biocompatibility [85]. Therefore, μPAD is increasingly being utilized in many fields, including POC diagnostics, environmental monitoring, medical diagnosis, and biochemical industries [81, 86].

Recently, tremendous efforts have also been made to develop a simple, convenient, fast, sensitive, and accurate μPAD that can diagnose COVID-19. Gong et al. reported instrument-free paper-based microfluidic enzyme-linked immunosorbent assay (ELISA) for quantitative IgA/IgM/IgG measurements. They accomplished blood-serum separation and detection of SARS-CoV-2-specific IgA/IgM/IgG antibodies via a pulling-force spinning top (PFST) combined with a paper-based microfluidic technique (Fig. 4a) [81]. This PFST-μPAD shows excellent sensitivity and specificity compared with traditional IgM/IgG detection and provides quantitative information on IgA/IgG/IgM antibodies. Another group also reported a SARS-CoV-2 antibody test using cellulose, an alternative membrane material, and a double-antigen sandwich format (Fig. 4b) [87]. They fabricated a three-dimensional channel and designed the device to have a constant flow rate. In addition, this μPAD diminished the background signal emanating from the human antibody in serum using functionalized SARS-CoV-2 antigens on behalf of anti-human antibodies. Using the cellulose-binding domain (CBD) conjugated SARS-CoV-2 nucleocapsid protein (NP) as the reporter reagent and enzyme-linked NP as the reporter reagent, the target antibody in clinical samples simultaneously immobilized on the cellulosic test zone within 15 min. In addition, Garneret et al. reported the μPADs for detecting SARS-CoV-2 RNA. They presented an easy-to-use portable device that combines isothermal nucleic acid amplification with paper microfluidics [88]. They constructed two different regions in μPAD for RNA extraction and RT-LAMP reaction. After RNA extraction from the RNA extraction membrane, the device was folded to transfer the extracted RNA to the reaction disk for the RT-LAMP reaction. The μPAD was tested for 21 clinical samples and showed remarkable detection sensitivity (1 copy/μL) and specificity (100%).

Meanwhile, an electrochemical paper-based analytical device (ePAD) has been introduced to increase detection sensitivity and obtain quantitative data. Most μPADs rely on

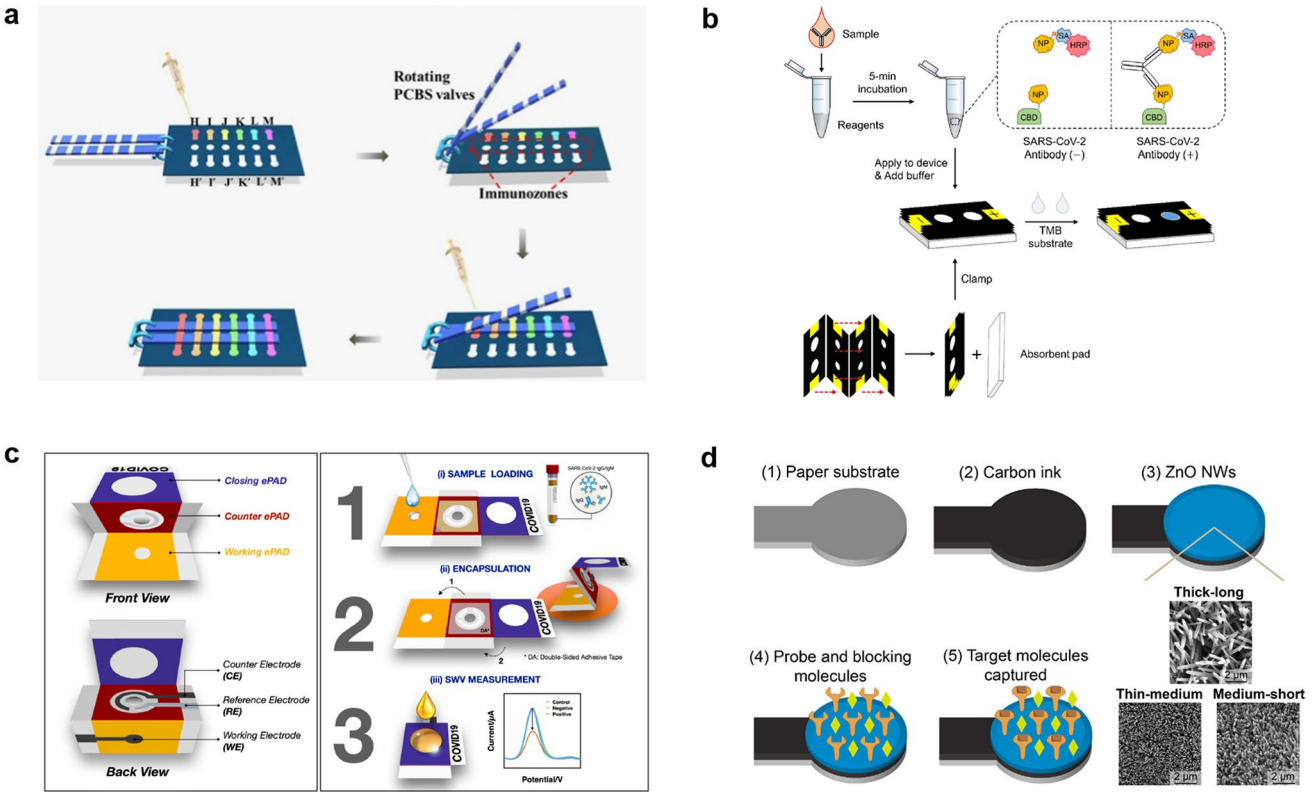

Fig. 4 **a** PFST-μPADs for quantitative SARS-CoV-2 IgA/IgM/IgG assay, reproduced with permission from [81], copyright 2021 ACS. **b** Three-dimensional μPADs for detecting SARS-CoV-2 specific antibodies based on affinity between cellulose and cellulose binding domain, reproduced with permission from [87], copyright 2021 ACS. **c** A label-free ePAD for detecting SARS-CoV-2-specific IgG and IgM antibodies, reproduced with permission from [56], copyright 2021 Elsevier. **d** A new ePAD-based COVID-19 diagnosis using ZnO NW-enhanced working electrode, reproduced with permission from [90], copyright 2021 Elsevier

optical readout by nanoparticle agglomeration, which often limits sensor performance [89]. The ePAD platforms have tremendous potential to discriminate small changes from the recognition events on the electrode surface with the label-free operation [56]. Yakoh et al. developed a label-free ePAD for detecting SARS-CoV-2-specific IgG and IgM antibodies. The ePAD comprises three parts; working ePAD, counter ePAD, and closing ePAD (Fig. 4c) [56]. A human serum sample was loaded to the test zone of the working ePAD, incubated, and washed. After the reaction, each component was manually assembled for electrochemical analysis. In the presence of SARS-CoV-2-specific antibodies, the current responses were decreased by interfering with the redox conversion of the redox indicator. This ePAD is capable of detecting targeted antibodies in clinical sera from patients with high sensitivity (100%) and can be extended to antigen detection. Also, another group presented a new ePAD-based COVID-19 diagnosis platform featuring zinc oxide nanowire (ZnO NWs) directly grown on working electrodes (Fig. 4d) [90]. They confirmed that the morphology and surface area of the ZnO NWs affected the sensing performance. The optimized ZnO NWs-enhanced working electrode successfully detected SARS-CoV-2-specific antibody (CR3022) even at a concentration of 10 ng/mL in human serum.

3.5 Commercial Tests in COVID-19 Pandemic

Most commercialized products are based on LFA technology. Although LFA has intrinsic drawbacks in terms of its performance (analytical sensitivity and reproducibility), LFA offers various advantages that outweigh these disadvantages. For example, it is rapid, low-cost (~ USD 0.1 – 2), has a POC test capability, has a long shelf life (up to 1–2 years), and requires small sample volumes. Furthermore, in the market, convenient and cost-effective diagnostic platforms are more attractive than expensive, time-consuming, and accurate platforms performed by trained professionals in well-equipped laboratories.

Table 1 summarizes EUA-approved commercialized antigen tests for the diagnosis of COVID-19. The table compares the types of tests, readout methods, target antigens, specimen types, and authorized sites for the tests. As shown in Table 1 and Fig. 5a, by April 2022, there were 50 EUA-approved commercial antigen test kits. Of these, LFA-based

Table 1 Emergency use authorization (EUA) approved antigen lateral flow tests for SARS-CoV-2

No.	Date EUA originally issued	Entity	Diagnostic (Most recent letter of authorization)	Readout	Target[1)]	Specimen	Authorized setting[2)]
1	2020.05.08	Quidel Corporation	Sofia SARS Antigen FIA	Fluorescence, Instrument read	N	Nasal swab	H, M, W
2	2020.07.02	Becton, Dickinson and Company (BD)	BD Veritor System for Rapid Detection of SARS-CoV-2	Instrument read	N	Nasal swab	H, M, W
3	2020.08.26	Abbott Diagnostics Scarborough, Inc	BinaxNOW COVID-19 Ag Card	Visual read	N	Nasal swab	H, M, W
4	2020.10.02	Quidel Corporation	Sofia 2 Flu + SARS Antigen FIA	Fluorescence, Instrument read	N	Nasal swab, Nasopharyngeal swab	H, M, W
5	2020.10.08	Access Bio, Inc	CareStart COVID-19 Antigen test	Visual read	N	Nasal swab, Nasopharyngeal swab	H, M, W
6	2020.12.07	Luminostics, Inc	Clip COVID Rapid Antigen Test	Instrument read	N	Nasal swab	H, M, W
7	2020.12.15	Ellume Limited	Ellume COVID-19 Home Test	Fluorescence, Instrument read (Bluetooth analyzer)	N	Nasal swab	Home, H, M, W
8	2020.12.16	Abbott Diagnostics Scarborough, Inc	BinaxNOW COVID-19 Ag Card Home Test	Visual read	N	Nasal swab	Home, H, M, W
9	2020.12.18	Quidel Corporation	QuickVue SARS Antigen Test	Visual read	N	Nasal swab	H, M, W
10	2021.02.04	Princeton BioMeditech Corp	Status COVID-19/Flu A&B	Visual read	N	Nasal swab, Nasopharyngeal swab	H, M, W
11	2021.03.01	Quidel Corporation	QuickVue At-Home COVID-19 Test	Visual read	N	Nasal swab	Home, H, M, W
12	2021.03.24	Becton, Dickinson and Company (BD)	BD Veritor System for Rapid Detection of SARS-CoV-2 & Flu A + B	Instrument read	N	Nasal swab	H, M, W
13	2021.03.31	Abbott Diagnostics Scarborough, Inc	BinaxNOW COVID-19 Ag 2 Card	Visual read	N	Nasal swab	H, M, W
14	2021.03.31	Abbott Diagnostics Scarborough, Inc	BinaxNOW COVID-19 Antigen Self Test	Visual read	N	Nasal swab	Home, H, M, W
15	2021.03.31	Quidel Corporation	QuickVue At-Home OTC COVID-19 Test	Visual read	N	Nasal swab	Home, H, M, W
16	2021.04.16	Celltrion USA, Inc	Celltrion DiaTrust COVID-19 Ag Rapid Test	Visual read	N, S	Nasopharyngeal swab	H, M, W
17	2021.05.06	InBios International, Inc	SCoV-2 Ag Detect Rapid Test	Visual read	N	Nasal swab	H, M, W
18	2021.05.20	Salofa Oy	Sienna-Clarity COVID-19 Antigen Rapid Test Cassette	Visual read	N	Nasopharyngeal swab	H, M, W
19	2021.06.04	OraSure Technologies, Inc	InteliSwab COVID-19 Rapid Test	Visual read	N	Nasal swab	Home, H, M, W
20	2021.06.04	OraSure Technologies, Inc	InteliSwab COVID-19 Rapid Test Rx	Visual read	N	Nasal swab	Home, H, M, W
21	2021.06.04	OraSure Technologies, Inc	InteliSwab COVID-19 Rapid Test Pro	Visual read	N	Nasal swab	H, M, W
22	2021.07.08	Ellume Limited	ellume.lab COVID Antigen Test	Fluorescence, Instrument read	N	Nasal swab	H, M, W
23	2021.07.13	GenBody Inc	GenBody COVID-19 Ag	Visual read	N	Nasal swab, Nasopharyngeal swab	H, M, W
24	2021.07.28	PHASE Scientific International, Ltd	INDICAID COVID-19 Rapid Antigen Test	Visual read	N	Nasal swab	H, M, W

Table 1 (continued)

No.	Date EUA originally issued	Entity	Diagnostic (Most recent letter of authorization)	Readout	Target[1)]	Specimen	Authorized setting[2)]
25	2021.08.02	Access Bio, Inc	CareStart COVID-19 Antigen Home Test	Visual read	N	Nasal swab	Home, H, M, W
26	2021.08.05	QIAGEN GmbH	QIAreach SARS-CoV-2 Antigen	Fluorescence, Instrument read	N	Nasal swab, Nasopharyngeal swab	H, M
27	2021.08.18	LumiraDx UK Ltd	LumiraDx SARS-CoV-2 Ag Test	Instrument read	N	Nasal swab, Nasopharyngeal swab	H, M, W
28	2021.08.24	Becton, Dickinson and Company (BD)	BD Veritor At-Home COVID-19 Test	Digital read (Image capture by smartphone app.)	N	Nasal swab	Home, H, M, W
29	2021.09.24	ANP Technologies, Inc	NIDS COVID-19 Antigen Rapid Test Kit	Visual read	N	Nasal swab	H, M, W
30	2021.10.04	ACON Laboratories, Inc	Flowflex COVID-19 Antigen Home Test	Visual read	N	Nasal swab	Home, H, M, W
31	2021.10.12	Xtrava Health	SPERA COVID-19 Ag Test	Visual read	N	Nasal swab	H, M, W
32	2021.10.21	Celltrion USA, Inc	Celltrion DiaTrust COVID-19 Ag Home Test	Visual read	N, S	Nasal swab, Nasopharyngeal swab	Home, H, M, W
33	2021.11.05	iHealth Labs, Inc	iHealth COVID-19 Antigen Rapid Test	Visual read	N	Nasal swab	Home, H, M, W
34	2021.11.22	InBios International Inc	SCoV-2 Ag Detect Rapid Self-Test	Visual read	N	Nasal swab	Home, H, M, W
35	2021.12.06	Nano-Ditech Corp	Nano-Check COVID-19 Antigen Test	Visual read	N	Nasal swab, Nasopharyngeal swab	H, M, W
36	2021.12.29	Siemens Healthineers	CLINITEST Rapid COVID-19 Antigen Self-Test	Visual read	N	Nasal swab	Home, H, M, W
37	2022.01.05	SD Biosensor, Inc	Pilot COVID-19 At-Home Test	Visual read	N	Nasal swab	Home, H, M, W
38	2022.01.14	iHealth Labs, Inc	iHealth COVID-19 Antigen Rapid Test Pro	Visual read	N	Nasal swab	H, M, W
39	2022.01.19	Maxim Biomedical, Inc	MaximBio ClearDetect COVID-19 Antigen Home Test	Visual read	N	Nasal swab	Home, H, M, W
40	2022.02.28	Oceanit Foundry LLC	ASSURE-100 Rapid COVID-19 Test	Visual read	N	Nasal swab	H, M, W
41	2022.03.16	PHASE Scientific International, Ltd	INDICAID COVID-19 Rapid Antigen At-Home Test	Visual read	N	Nasal swab	Home, H, M, W
42	2022.04.06	Xiamen Boson Biotech Co., Ltd	Rapid SARS-CoV-2 Antigen Test Card	Visual read	N	Nasal swab	Home, H, M, W
43	2022.04.06	OSANG LLC	OHC COVID-19 Antigen Self Test	Visual read	N	Nasal swab	Home, H, M, W

[1)] N: Nucleocapsid protein

S: Receptor binding domains (RBDs) from the spike proteins

[2)] H: Laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform high complexity tests

M: Laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform moderate complexity tests

W: Patient care settings operating under a CLIA Certificate of Waiver

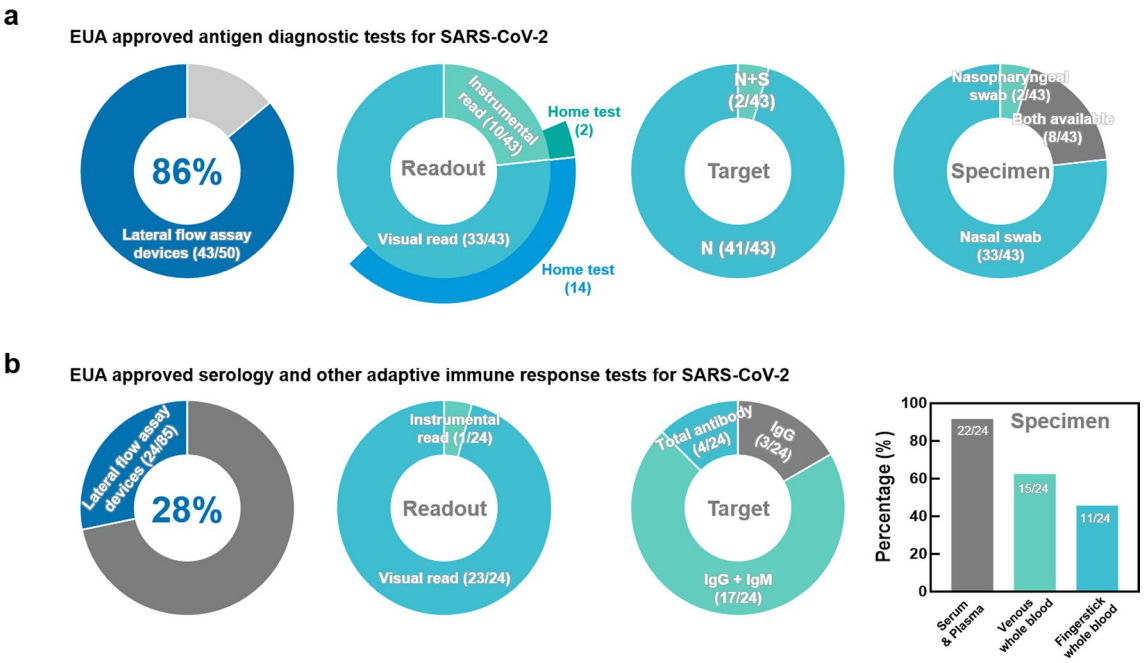

Fig. 5 Development trends of EUA-approved commercialized antigen (a) and serological tests (**b**) for the diagnosis of COVID-19. **a** To satisfy the demand for high-throughput testing, LFA-type diagnostic tests have been mainly developed. For rapid, convenient, and cost-effective diagnosis, the kits are visually readable and developed for testing using nasal swab samples. **b** LFA-based serology kits accounted for only 28% of total products due to the low sensitivity. Most LFA-based serology tests visually confirm the results and target two or more antibodies

diagnostic tests account for 86%. To satisfy the demand for high-throughput testing due to COVID-19, many diagnostic companies are currently focusing on their efforts to develop rapid and cost-effective diagnostic kits. Moreover, the visual reading method is the primary method to confirm the signal, and other methods include using a reader to read the fluorescent signal. Most kits mainly target N protein, which is known to exist in abundance, and some kits target both N and S for diagnosing COVID-19. Generally, the nasopharyngeal swab method is the preferred method for specimen collection in COVID-19. However, the Centers for Disease Control and Prevention (CDC) supports all methods. The nasal swab method is less uncomfortable than the nasopharyngeal swab method; therefore, many diagnostic kits have been developed for nasal swab samples.

Table 2 provides a collection of currently available EUA-approved serological tests for the diagnosis of COVID-19. The table compares the test types, target immunoglobulins, readout method, specimen type, and authorized sites for the tests. Unlike antigen test kits, LFA-based serology kits accounted for only 28% of total products (Fig. 5b). Many serological tests utilizing ELISA and chemiluminescence immunoassays (CLIA) are available under EUA-approval. However, most (except for one) EUA-approved LFA-based serological tests confirm the presence or absence of SARS-CoV-2-specific antibodies by visual readout. Furthermore, commercialized serological tests target the total antibodies (4/24) or dual antibodies, IgM + IgG (17/24), to improve detection accuracy. In addition, most kits perform serological tests using serum and plasma specimens. However, diagnostic kits using fingerstick whole blood are also on the rise to increase the convenience of use and utility of POC diagnosis.

On the other hand, the FDA has withdrawn EUA approval if the diagnostic performance of the kits is insufficient. For example, several products, such as COVID-19 Direct Antigen Rapid Test (E25Bio, Inc.) and SARS-CoV-2 Antigen Rapid Test (Colloidal Gold) (Skippack Medical Lab, LLC), were previously EUA-approved, but are currently excluded from the list of EUA-approved kit. Moreover, Flowflex COVID-19 Anti-Tigen Home Tests (#30 in Table 1) and the Celltrion DiaTrust COVID-19 Ag Rapid Tests (#32 in Table 1) have been recalled and restricted the use of the tests (only some products with specific lot numbers). In addition, COVID-19 At-Home Test (SD Biosensor, Inc.) has been withdrawn from EUA approval, but this product has been revised and reissued EUA approval with the changed name; Pilot COVID-19 At-Home Test (#37 in Table 1).

Table 2 Emergency use authorization (EUA) approved antibody lateral flow tests for SARS-CoV-2

No.	Date EUA originally issued	Entity	Diagnostic (most recent letter of authorization)	Target	Readout	Specimen	Authorized setting
1	2020.09.03	Sugentech, Inc	SGTi-flex COVID-19 IgG	IgG	Visual read	Serum, Plasma, Venous whole blood, Fingerstick whole blood	H, M, W
2	2021.01.11	ADVAITE, Inc	RapCov Rapid COVID-19 Test	IgG	Visual read	Fingerstick whole blood	H, M, W
3	2021.08.24	InBios International, Inc	SCoV-2 Detect IgG Rapid Test	IgG	Visual read	Serum, Plasma, Venous whole blood, Fingerstick whole blood	H, M, W
4	2020.05.29	Healgen Scientific LLC	COVID-19 IgG/IgM Rapid Test Cassette (Whole Blood/Serum/Plasma)	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood	H, M
5	2020.06.04	Hangzhou Biotest Biotech Co., Ltd	RightSign COVID-19 IgG/IgM Rapid Test Cassette	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood, Fingerstick whole blood	H, M, W
6	2020.06.18	Biohit Healthcare (Hefei) Co. Ltd	Biohit SARS-CoV-2 IgM/IgG Antibody Test Kit	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood	H, M
7	2020.06.19	Hangzhou Laihe Biotech Co., Ltd	LYHER Novel Coronavirus (2019-nCoV) IgM/IgG Antibody Combo Test Kit (Colloidal Gold)	IgM, IgG	Visual read	Serum, Plasma	H, M
8	2020.07.06	Assure Tech. (Hangzhou Co., Ltd)	Assure COVID-19 IgG/IgM Rapid Test Device	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood, Fingerstick whole blood	H, M, W
9	2020.07.13	Salofa Oy	Sienna-Clarity COVIBLOCK COVID-19 IgG/IgM Rapid Test Cassette	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood, Fingerstick whole blood	H, M, W
10	2020.07.17	Megna Health, Inc	Rapid COVID-19 IgM/IgG Combo Test Kit	IgM, IgG	Visual read	Serum, Plasma, Fingerstick whole blood	H, M, W
11	2020.07.24	Access Bio, Inc	CareStart COVID-19 IgM/IgG	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood, Fingerstick whole blood	H, M, W
12	2020.07.24	Xiamen Biotime Biotechnology Co., Ltd	BIOTIME SARS-CoV-2 IgG/IgM Rapid Qualitative Test	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood	H, M
13	2020.08.25	Biocan Diagnostics Inc	Tell Me Fast Novel Coronavirus (COVID-19) IgG/IgM Antibody Test	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood	H, M
14	2020.08.31	TBG Biotechnology Corp	TBG SARS-CoV-2 IgG / IgM Rapid Test Kit	IgM, IgG	Visual read	Serum, Plasma	H, M
15	2020.09.23	Jiangsu Well Biotech Co., Ltd	Orawell IgM/IgG Rapid Test	IgM, IgG	Visual read	Serum, Plasma	H, M

Table 2 (continued)

No.	Date EUA originally issued	Entity	Diagnostic (most recent letter of authorization)	Target	Readout	Specimen	Authorized setting
16	2020.09.29	Nirmidas Biotech, Inc	Nirmidas COVID-19 (SARS-CoV-2) IgM/IgG Antibody Detection Kit	IgM, IgG	Visual read	Serum, Plasma	H, M
17	2020.11.23	Innovita (Tangshan) Biological Technology Co., Ltd	Innovita 2019-nCoV Ab Test (Colloidal Gold)	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood	H, M
18	2020.12.15	ACON Laboratories, Inc	ACON SARS-CoV-2 IgG/IgM Rapid Test	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood	H, M
19	2020.12.31	Nirmidas Biotech, Inc	MidaSpot COVID-19 Antibody Combo Detection Kit	IgM, IgG	Visual read	Serum, Plasma, Fingerstick whole blood	H, M, W
20	2021.06.24	Access Bio, Inc	CareStart EZ COVID-19 IgM/IgG	IgM, IgG	Visual read	Serum, Plasma, Venous whole blood, Fingerstick whole blood	H, M, W
21	2020.07.10	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd	WANTAI SARS-CoV-2 Ab Rapid Test	Total Antibody	Visual read	Serum, Plasma, Venous whole blood	H, M
22	2021.05.11	QIAGEN, GmbH	QIAreach Anti-SARS-CoV-2 Total Test	Total Antibody	Digital read	Serum, Plasma	H, M
23	2021.05.24	NOWDiagnostics, Inc	ADEXUSDx COVID-19 Test	Total Antibody	Visual read	Serum, Plasma, Venous whole blood, Fingerstick whole blood	H, M, W
24	2021.06.04	Diabetomics, Inc	CovAb SARS-CoV-2 Ab Test	Total Antibody	Visual read	Oral fluid	H, M, W

4 Advanced Technologies in Paper-Based Diagnostic Platform

LFA-based diagnostic tests have great potential in the COVID-19 pandemic; for more effective control the COVID-19 pandemic, unresolved improvements in LFA performance are required. In general, LFA-based rapid diagnostic tests focus on reducing costs and time, extending test frequency, and verifying test results in the field. However, it did not show satisfactory performance in terms of detection accuracy, as evaluated by sensitivity and specificity. Particularly, LFA-based rapid diagnostic tests may produce false-negative results during the early stage of infection because typical LFA tests have a relatively high detection limit compared to RT-qPCR. Additionally, the sensitivity of the kit may be low when testing asymptomatic or mildly symptomatic individuals. Some studies have shown that the clinical assay accuracy of commercial rapid diagnostic kits is well below the company's claimed sensitivity (87–97.5%) and specificity (100%) (positive predictive value: 11–50%) [91]. Continuous improvements (high sensitivity and specificity to reduce false-negative/positive predictive results) of LFA-based rapid diagnostic tests are essential for accurate POC testing of COVID-19.

4.1 Nanomaterial-Based Paper-Based Diagnostic Platform

Unlike molecular methods, paper-based antigen/antibody tests do not amplify the target molecules; thus, the detection sensitivity of these tests depends on the quantity of analytes and their signals. Signal enhancement is the best way to improve the sensitivity of an LFA and can be achieved by developing a new optical reporter and utilizing an external signal reader [92]. Typical LFA tests use gold nanoparticles as signal reporters; however, their sensitivities are not very high. Extensive efforts have been made to increase diagnostic sensitivity by improving the performance of signaling molecules in various studies. Liu et al. reported an advanced LFA based on gold nanoparticles for enhanced specific binding and thermal contrast amplification (TCA) for signal amplification. With TCA, the gold nanoparticles captured in the test line were excited by laser irradiation and exhibited a substantial photothermal effect, enabling the detection

of subvisual positives. They successfully detected SARS-CoV-2 receptor-binding domain (RBD) antigen as low as 28.6 aM in a human nasopharyngeal swab [93]. Selenium nanoparticles exhibit favorable biocompatibility and are readily conjugated with biological molecules without losing their activity [94]. A POC selenium nanoparticle-based LFA was developed to detect SARS-CoV-2 IgM and IgG [72]. In this study, the authors made a new selenium nanoparticle-based LFA kit and visually detected anti-SARS-CoV-2 IgG and IgM antibodies in human serum within 10 min. Furthermore, the authors performed a clinical evaluation using a sample of 90 patients with COVID-19 and 263 uninfected negative controls, demonstrating a sensitivity and specificity of 93.33% and 97.34%, respectively. This selenium nanoparticle-based LFA showed superior detection limits compared to the gold nanoparticle-based LFA in IgM antibody detection and did not show cross-reactivity with influenza A, influenza B, anti-nuclear antibodies, and rheumatoid factor. On the other hand, in recent years, quantum dots (QDs) have been widely used as fluorescent signal reporters in LFA because of their excellent optical properties, such as quantifiable fluorescence intensity, broad excitation, and high light stability [95–98]. Wang et al. reported a dual-mode QD-based LFA for the detection of SARS-CoV-2-specific IgM and IgG antibodies (Fig. 6a). This assay was validated using 16 positive serum samples from patients with COVID-19 and 41 negative control samples and achieved 100% sensitivity and 100% specificity [99]. Typical fluorescent dyes suffer from a narrow Stokes shift (20–30 nm), photobleaching, and low emission intensity, which cause reduced sensitivity and dye stability issues [100–102]. Chen et al. developed a rapid and sensitive LFA that uses lanthanide chelate-encapsulated polystyrene nanoparticle-based LFA to detect SARS-CoV-2-specific IgG antibodies (Fig. 6b) [103]. Seven positive and 12 negative control samples from patients with COVID-19 were tested, and they obtained meaningful results consistent with those obtained by RT-qPCR (except for one negative case).

4.2 Aptamer-Based Paper-Based Diagnostic Platform

Aptamers are DNA or RNA molecules capable of binding to a wide range of molecules with high affinity and specificity, selected by the systematic evolution of ligands by

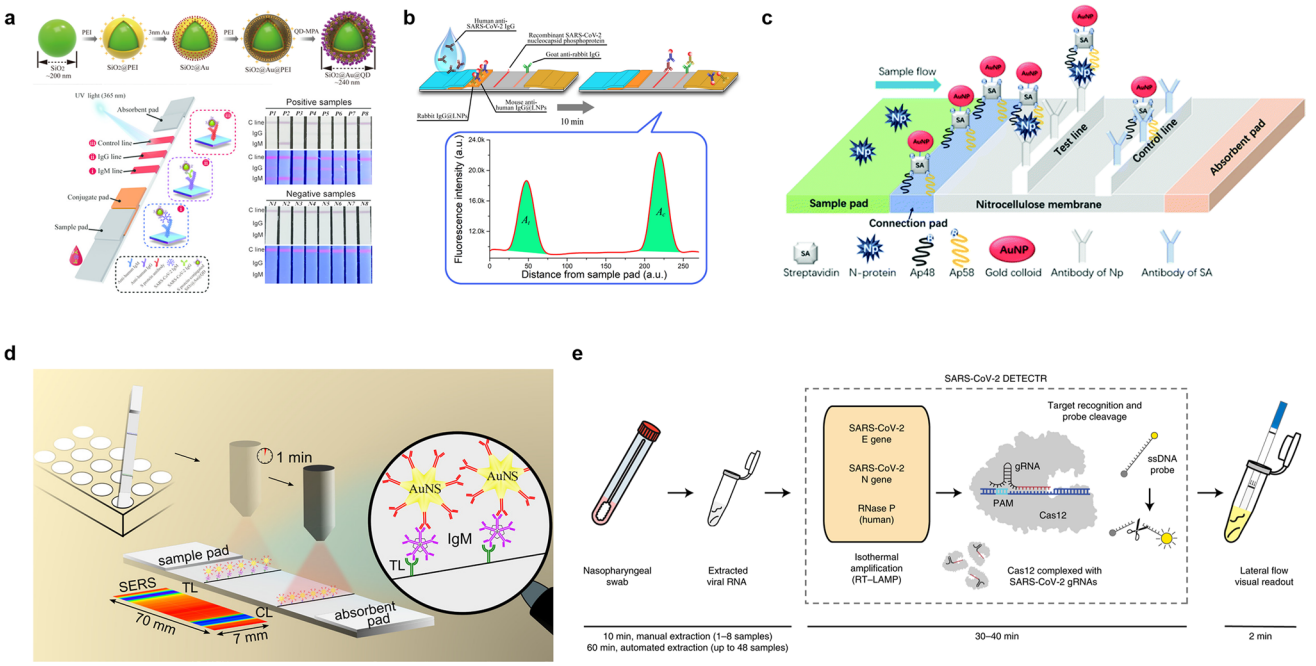

Fig. 6 **a** Fabrication process of the dual-mode SiO2@Au@QD and schematic of dual-mode LFA for detecting anti-SARS-CoV-2 IgM and IgG antibodies, reproduced with permission from [99], copyright 2020 ACS. **b** Lanthanide-doped nanoparticles-based LFA for detecting anti-SARS-CoV-2 IgG, reproduced with permission from [103], copyright 2020 ACS. **c** DNA aptamers-based LFA for detecting SARS-CoV-2 N antigen, reproduced with permission from [111], copyright 2020 RSC. **d** SERS-based LFA for SARS-CoV-2-specific IgM detection, reproduced with permission from [122], copyright 2021 ACS. **e** Workflow of CRISPR-based DETECTR assay. The SARS-CoV-2 DETECTR comprises RNA extraction, RT-LAMP, Cas12 detection, and LFA, reproduced with permission from [129], copyright 2020 Nature research

exponential enrichment (SELEX) technology [104]. Aptamers recognize viral proteins with high sensitivity and specificity, enabling the rapid diagnosis of intractable infectious diseases and their use as antiviral agents [105, 106]. For the detection of SARS-CoV-2-specific antigens, some aptamers have already been identified [107–110], and studies to apply these aptamers to LFA are being conducted. Zhang et al. first reported DNA aptamer-based LFA targeting COVID-19 diagnosis (Fig. 6c) [111]. They discovered DNA sequences with high binding affinity for the SARS-CoV-2 N protein and constructed aptamer pairs for sandwich immunoassays. They demonstrated that LFA could detect even tens of pM levels of N proteins using these aptamers. In contrast, an aptamers-based rapid COVID-19 test for targeting the S protein of SARS-CoV-2 was also developed. Kacherovsky et al. demonstrated novel DNA aptamers that bind to the spike N-terminal domain of SARS-CoV-2 with a high specificity and affinity (< 80 nM). They applied these aptamers in LFA to detect inactivated SARS-CoV-2 at concentrations as low as 5×10^5 copies/mL [112]. Recently, aptamers have been extensively studied as biorecognition molecules for LFAs. In these studies, various signal amplification strategies, such as enzyme amplification, magnetic enrichment, modification of gold nanoparticles, and integration with isothermal amplification, have been exploited [113–116]. Therefore, this strategy can be fully utilized for diagnosing COVID-19, and the aptamer-based COVID-19 LFA technology will become more mature in the future.

4.3 SERS-Based Paper-Based Diagnostic Platform

Surface-Enhanced Raman spectroscopy (SERS) is a powerful vibrational spectroscopy technique that allows sensitive, specific, quantitative, and multiplex detection with a high enhancement factor. Even considering the many advantages of SERS, the technology is still limited in its application to point-of-care (POC) testing. SERS requires specific substrates made of metallic nanomaterials with multiple active sites ("hot spots") that enhance the Raman signal. The commercialization of SERS substrates is practically challenging due to the difficulty of manufacturing cost-effective and highly reproducible substrates [117, 118]. Also, the integration of a portable Raman reader into a general diagnostic system for POC testing is another significant issue [119]. Also, due to the complexity of the signal, it is almost impossible to intuitively interpret the results in the field, except for a trained person [120]. The use of SERS in combination with LFA is an upcoming platform for providing reliable and accurate results with the advantage of high throughput and POC applicability. The use of SERS in combination with LFA is an upcoming platform for providing reliable and accurate results with the advantage of high throughput. One novel study was the first to demonstrate an SERS-based LFA for the simultaneous detection of SARS-CoV-2-specific IgM/IgG antibodies. High-sensitivity analysis of the target IgM and IgG antibodies was accomplished using novel SERS tags labeled with dual layers of Raman dye (SiO2@Ag) [121]; the LOD was 800 times lower than the visual readout. They also validated the clinical feasibility of this diagnostic platform using 19 positive serum samples from patients with COVID-19 and 49 negative serum samples from healthy controls. Moreover, another group demonstrated the diagnostic performance of SERS-based LFA for SARS-CoV-2-specific IgM/IgG antibodies compared with conventional LFA (Fig. 6d) [122]. In this study, the authors confirmed that the sensitivity of SERS-based LFA (100 fg/mL) was seven orders of magnitude higher than that of naked-eye detection (1 μg/mL) for detecting IgM antibodies in a buffer. Similar studies [123, 124] have been performed by altering SERS tags or plasmonic nanomaterials, and in all these studies, the sensitivity of SERS-based LFAs was significantly improved compared to that of conventional LFAs.

4.4 CRISPR-Cas system-based diagnostic platform

The CRISPR/Cas9 system is widely recognized as an adaptive immune defense system that can resist foreign genetic material in most prokaryotes. Owing to its unique sequence-specific properties, CRISPR/Cas technology has recently attracted increasing interest in biosensing [125, 126]. Although several highly sensitive and POC-applicable RNA detection methods, such as reverse transcription-recombinase polymerase amplification (RT-RPA) and reverse transcription-loop-mediated isothermal amplification (RT-LAMP), have been proposed, these methods suffer from non-specific amplification under isothermal conditions, leading to false-positive results. Integration of isothermal amplification techniques with CRISPR can significantly decrease the risk of non-specific detection [127, 128]. Taking advantage of these properties, many CRISPR-coupled isothermal amplification techniques have been developed to efficiently amplify target genes and improve the performance of COVID-19 detection.

Broughton et al. developed a rapid (< 40 min), easy-to-implement, and accurate CRISPR–Cas12-based lateral flow assay to detect SARS-CoV-2, called SARS-CoV-2 DNA endonuclease-targeted CRISPR trans reporter (DETECTR) (Fig. 6e) [129]. The SARS-CoV-2 DETECTR comprises RT-LAMP for RNA extraction and gene amplification, Cas12 detection of predefined viral sequences, and confirmation of the detection of viral RNA by cleavage of reporter molecules. The CRISPR-based DETECTR was validated with clinical samples (36 samples from patients with COVID-19 and 42 samples from patients with other viral respiratory infections), showing a 95% positive predictive agreement and 100% negative predictive agreement.

Patchsung et al. also demonstrated another CRISPR-based LFA, SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) [127]. They reported clinical validation of the two-step CRISPR-Cas13-based SHERLOCK system (first step: RT-RPA to isothermally amplify the viral gene, second step: CRISPR-Cas-mediated detection of the amplified genes using Cas13a from *Leptotrichia wadei*) for sensitive and specific detection of SARS-CoV-2 RNA. In the clinical evaluation of the diagnostic platform, they found that the SHERLOCK detection system for SARS-CoV-2 RNA extracted from nasopharyngeal and throat swabs from patients infected with SARS-CoV-2 showed 100% specificity, with 96% sensitivity for fluorescence readout and 88% sensitivity for LFA readout. Other groups have also demonstrated novel CRISPR-based LFAs, such as SHINE (simplified highlighting of infection for epidemiological detection) [130] and BioSCAN (biotin-binding-specific CRISPR-based assay for nucleic acid detection) [131]. These CRISPR-based LFAs successfully detected SARS-CoV-2 RNA with high sensitivity and specificity, confirming their applicability in clinical sample testing.

5 Conclusions and Future Perspectives

Paper is a readily accessible and inexpensive material; therefore, the mass production of paper-based biosensors would be relatively low in cost. Moreover, because paper is inherently able to transport fluidic samples via capillary flow, the use of pumps or other external equipment to drive fluid flow is unnecessary. Moreover, various paper functionalization methods have already been established, and paper-based biosensors can be widely used in various applications of POC diagnostic systems, making them the most attractive diagnostic systems in the market. Timely diagnosis, effective treatment, and future prevention are the most critical factors for successfully managing COVID-19. Rapid and accurate diagnosis is the first step in preventing and controlling the COVID-19 epidemic. Various rapid and cost-effective POC tests, including LFA-based diagnostic tests, have been developed as powerful and effective methods to control the COVID-19 outbreak and have contributed significantly to the rapid identification of new infections and implementation of quarantine measures.

Although the LFA-based test has been used to complement the current gold standard technique, RT-qPCR, paper-based analytical devices have great potential to provide POC diagnostic systems to developing countries or to prepare them for another outbreak of disease X that may occur after COVID-19. In addition, intensive efforts are being made in academia and industry to address some of the remaining challenges, including the integration of new technologies such as nanotechnology, aptamers, SERS, and CRISPR/Cas systems.

As we progress through the COVID-19 pandemic, analysis techniques for the existing virus have rapidly evolved and improved to become more sensitive, automated, faster, and with higher performance [13]. However, there is still an aperture that needs to be addressed to develop an ideal and universal POC diagnostic platform. Smartphones, which offer imaging, filtering, and image/data processing, can be powerful tools to compensate for current POC diagnostic platforms. Because many people always carry their smartphones, they can effectively serve as handheld readers that rapidly and accurately check for infections and contribute significantly to disease control and surveillance. In addition, it is expected that diagnosis performance will be improved through the introduction of artificial intelligence (AI). Recently, the potential application of AI-based COVID-19 diagnosis has been extensively explored in the field of lung detection imaging, such as computed tomography (CT) imaging [132, 133], chest radiographs (X-ray) imaging [134, 135]. By learning from tremendous amounts of diagnostic results through deep learning techniques and using them to present accurate current or future results, more effective infectious disease management is possible.

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Declarations

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

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