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On the Challenges for the Diagnosis of SARS-CoV-2 Based on a Review of Current Methodologies

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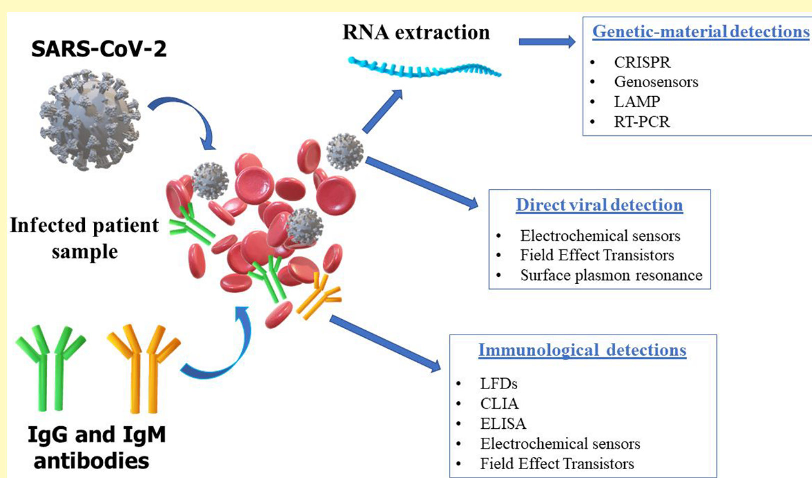

ABSTRACT: Diagnosis of COVID-19 has been challenging owing to the need for mass testing and for combining distinct types of detection to cover the different stages of the infection. In this review, we have surveyed the most used methodologies for diagnosis of COVID-19, which can be basically categorized into genetic-material detection and immunoassays. Detection of genetic material with real-time polymerase chain reaction (RT-PCR) and similar techniques has been achieved with high accuracy, but these methods are expensive and require time-consuming protocols which are not widely available, especially in less developed countries. Immunoassays for detecting a few antibodies, on the other hand, have been used for rapid, less expensive tests, but their accuracy in diagnosing infected individuals has been limited. We have therefore discussed the strengths and limitations of all of these methodologies, particularly in light of the required combination of tests owing to the long incubation periods. We identified the bottlenecks that prevented mass testing in many countries, and proposed strategies for further action, which are mostly associated with materials science and chemistry. Of special relevance are the methodologies which can be integrated into point-of-care (POC) devices and the use of artificial intelligence that do not require products from a well-developed biotech industry.

KEYWORDS: *SARS-CoV-2, point-of-care, COVID-19 diagnosis, biosensors, lateral flow devices, RT-PCR, surface plasmon resonance, nanoparticles*

The COVID-19 pandemic has shown the relevance of developing new tools for diagnosis, especially with low-cost technologies that permit rapid assays within the so-called *point-of-care* (POC) diagnosis paradigm.[1] The existence of well-established diagnostic methodologies for detecting viral genetic material[2,3] and human antibodies using rapid tests[4,5] has made it possible to achieve a relatively early detection of COVID-19 infection, in some cases with high accuracy. This has been instrumental for governments and societies to take proper actions to control spread and minimize the overall damage. Indeed, mass testing has been recommended from the early days of the pandemic by the World Health Organization (WHO)[6] for the surveillance and control of the spread of the disease. There are, however, important challenges to be faced in terms of performance of the diagnostic tools for detecting both genetic material and antibodies, mostly due to the cost and testing speed. Even more importantly, only a few countries could fully benefit from the existing technology, either because

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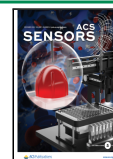

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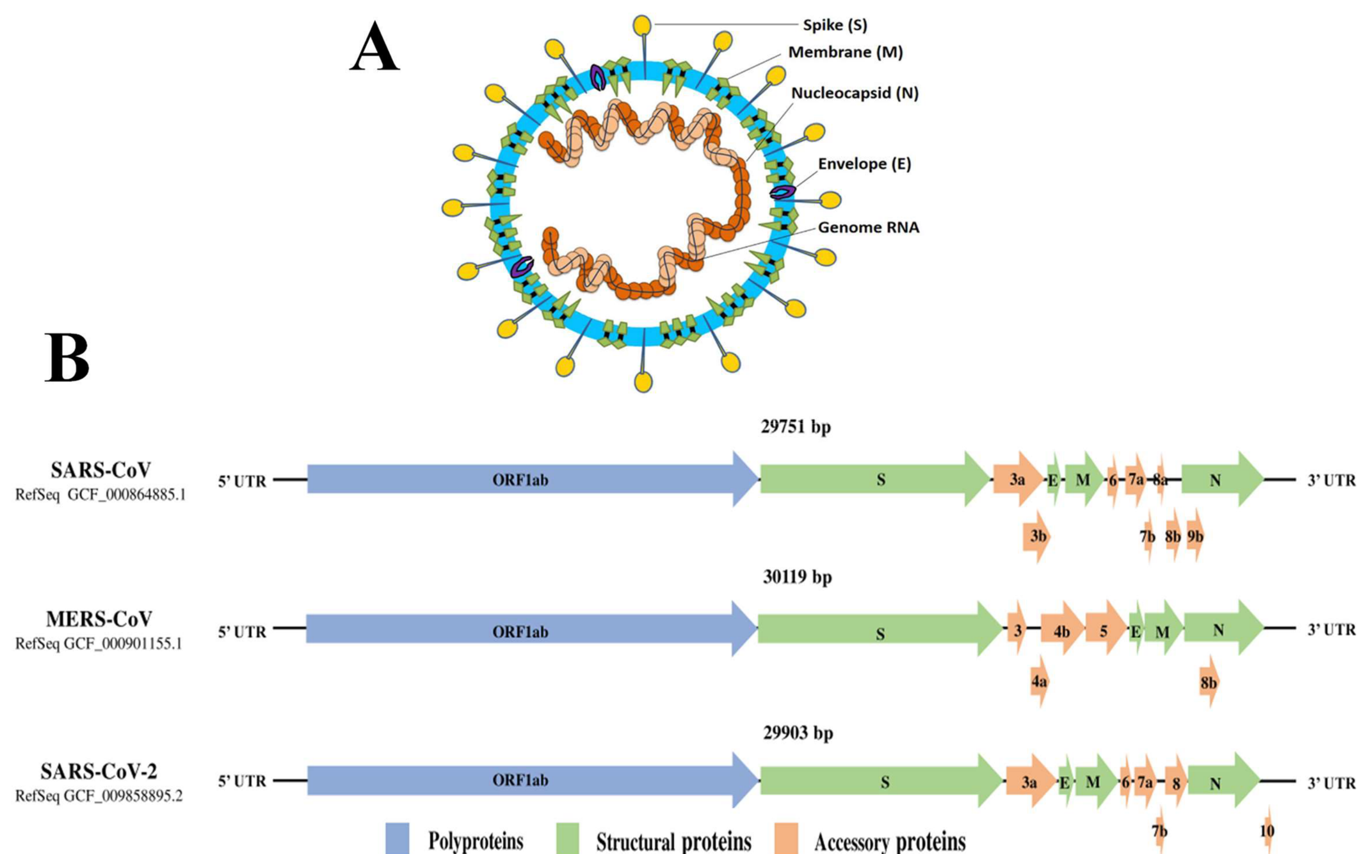

Figure 1. (A) Representation of CoV structure containing its spike glycoprotein (S), envelope protein (E), nucleocapsid protein (N), transmembrane glycoprotein (M), and its RNA viral genome. Reprinted with permission from ref 32. Copyright 2020, John Wiley and Sons. (B) Genome structure of SARS-CoV, MERS-CoV, and SARS-CoV-2 and encoded proteins. Reprinted with permission from ref 22. Copyright 2020, John Wiley and Sons.

the methods are too expensive or not easily deployable in poorer settings. Overcoming these challenges by using efficient and ready-to-use biosensor workflow research products could rapidly address the outbreak, as we shall elaborate upon in this article.

The failure of many countries to implement mass testing during the COVID-19 pandemic has highlighted the need for extra efforts and investments in the research and technology development of disease diagnosis. This is crucial to guarantee the security of humanity in general and of nations in view of possible outbreak of other pandemics. Here, we propose three different strategies to be adopted by governments and the scientific community for future epidemics to ensure protection of the population: (i) dissemination of plants in the biotechnology industry or at least ensure means to adequately supply the diagnostic tools to all countries, (ii) development of low-cost alternatives for the detection of genetic materials and immunoassays, particularly within the paradigm of POC diagnosis, and (iii) development of diagnostic strategies based on pattern recognition methods, as this minimizes the limitations brought by the lack of biotechnology industry. To achieve these goals, we suggest readaptation of existing methodologies for the diagnosis of COVID-19. This will make it possible to monitor current and future infectious diseases and place humankind in better shape to combat upcoming outbreaks of pathogenic diseases.

In this review, we mainly focus on the genosensing and immunosensing technologies which could be adapted to COVID-19 diagnosis, in addition to the molecular diagnosis and diagnosis exploiting pattern recognition. Attempts are made to provide a comprehensive review of the possibilities for diagnosis through illustration of the potential of such technologies. This review article is organized as follows. First, we comment on the structure, receptors, and possible targets of SARS-CoV-2 since an effective diagnosis requires understanding of the molecular machinery of the pathogens. The SARS-CoV-2 Diagnosis section introduces a brief discussion of the different detection techniques employed for COVID-19, with emphasis on the need to leverage distinct types of detection. An overview is presented of the challenges in detecting SARS-CoV-2 with nucleic acids-based and immuno-based techniques in the next two sections. Before concluding, we discuss emerging technologies that hold promise for the near future in the section Emerging Strategies for Diagnosis of COVID-19.

STRUCTURE OF SARS-COV-2

Coronaviruses (CoVs) belong to the *Coronaviridae* family of enveloped positive-stranded RNA viruses that exhibit the largest RNA genome of all known viruses. This family is divided into four main genera: alpha, beta, gamma, and delta-CoVs.[7,8] CoVs can be hosted by birds (gamma, delta-CoVs) or mammals (alpha, beta-CoVs), causing intestinal and respiratory illnesses.[8,9] Before the emergence of the severe acute respiratory syndrome coronavirus (SARS-CoV-2) in the outbreak of Covid-19 in December, 2019,[10] only six CoV

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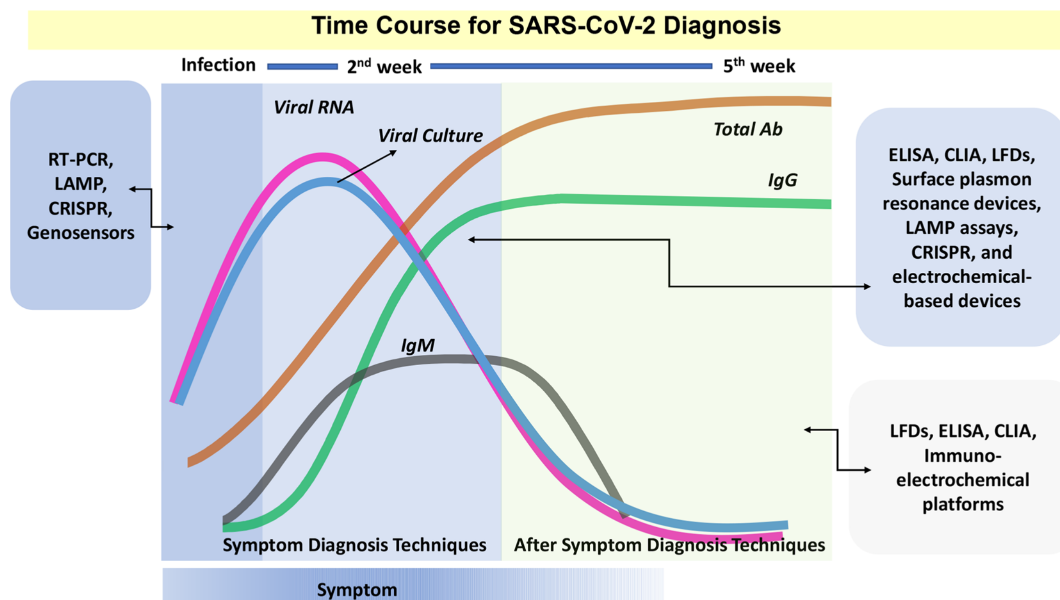

Figure 2. SARS-CoV-2 most appropriate detection methods along the course of infection. This figure is an illustrative scheme and it should be mentioned that discrepancies exist in the literature, especially for the tails of the curves. We decided to keep the qualitative character until more data are collected and a consensus is established on the time dependences.

species were known to infect humans. Four of these, i.e., HCoV-NL63, HCoV-HKU1, HCoV-OC43, and HCoV-229E, are related to mild respiratory infections,[8,11] while SARS-CoV and MERS-CoV cause severe respiratory illnesses. SARS-CoV caused an endemic in 2002−2003 in Guangang, China, and MERS-CoV emerged in Saudi Arabia in 2012.[8,11] Both SARS-CoV and MERS-CoV spread to several countries, infecting thousands of individuals with respiratory and neurological diseases with a high mortality rate.[12] The recent seventh member, SARS-CoV-2, is highly pathogenic. The disease it causes, referred to as COVID-19, is much more infectious and has spread to more than 200 countries in a time span of less than six months. Although the elderly are the most severely affected, with up to 50% of fatalities, a large number of hospitalizations have occurred for adult healthy patients with 2−11% fatality rate.[13] As of 14th October, 2020, more than 38 million people were infected by SARS-CoV-2 with at least one million deaths confirmed.[14]

The severity of the COVID-19 outbreak has led to a global mobilization by the pharmaceutical industry, governments, and academia to develop efficient diagnostics for mass testing, create a safe vaccine, and investigate treatments based on already approved medications.[15,16] In all of these endeavors, structural studies of SARS-CoV-2 are proving essential.[17−21] All CoVs exhibit similar structures with their genomes arranged in a similar fashion, as illustrated in Figure 1A. The viral genome and the nucleocapsid protein (N) are complexed to form a helical case within the hemagglutinin−esterase viral membrane (this latter is only present in some beta-CoVs). The viral gene also encodes a spike protein (S), nucleocapsid (N), internal protein (I), small membrane envelope protein (E), and a membrane protein (M).[7] In addition, a 5′-untranslated region (UTR), a 3′ UTR, nonstructural open reading frames (ORFs), and a conserved replicase domain (ORF1ab) (Figure 1B) exist in the viral genome.[22] The spike protein of SARS-CoV-2 is divided into the subunits S1 and S2, with a functional polybasic furin cleavage at the S1−S2 boundary, which can improve infection in host cells.[11,23] The spike protein receptor binding domain (RBD) localized in S1[24] has six amino acids (N501, L455, Q493, F486, S494, and Y505)[11] that are essential for binding on human ACE2 (Angiotensin-Converting Enzyme 2) receptors.[19] Among these six amino acids, five differ from SARS-CoV to SARS-CoV-2.[11] This interaction between the spike protein RBD and ACE2 receptors, which is crucial for the high contamination rate of SARS-CoV-2 in comparison to other human CoVs, has been studied in detail,[18,19,25−27] and is a potential therapeutic target. The SARS-CoV-2 spike protein may be useful for neutralization immunoassays[28] and a target for POC tests. The remaining structural proteins, N, E, and M, are mostly involved in the regulatory functions, RNA synthesis, protective function against the host immune system, and viral pathogenesis. These are more conserved proteins as compared to the S among the several human CoVs known so far. Moreover, structural similarities in SARS-CoV, MERS-CoV, and SARS-CoV-2 open the possibility for application or adaptation of existing diagnosis technologies and efficient treatment of COVID-19. On the other hand, SARS-CoV-2 exhibits a diverse gene position and has the chance of continued variation in the genome sequence due to the pandemic-scale spread of the disease.[29,30] Previous studies on several genomic sequences of SARS-CoV-2 revealed approximately 4% genomic mutation of total 220 strains analyzed,[31] suggesting the coexistence of different strains, which might be a new challenge for several diagnostic methods.

■ SARS-COV-2 DIAGNOSIS

SARS-CoV-2 has a unique biological characteristic, which brings several challenges to the health systems globally, and resulted in a poor response to contain the pathogenic disease. After the genetic sequence of the virus was known, the disease could be diagnosed with molecular testing based on viral RNA, such as reverse trancriptase polymerase chain reaction (RT-PCR), which are laboratory based and required skilled persons for operating sophisticated equipment. Therefore, mass testing could not be performed in the beginning of the pandemic due

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to the unavailability of testing facilities to the general public. Thanks to the research and development efforts of the clinical laboratory and academic researchers, several new and modified diagnostic tools were developed to make them available at varied locations. Indeed, today several types of diagnostic tools are available for detecting SARS-CoV-2.

Detection of SARS-CoV-2 has been made with various analytical techniques, either through quantification of nucleic acids or by measuring the immunoresponse of infected humans via antibody detection. The genetic-based techniques comprise RT-PCR, LAMP (Loop-Mediated Isothermal Amplification), CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), and genosensors, while immuno-based assays are mainly made with LFDs (Lateral Flow Devices), CLIA (Chemiluminescent Immunoassays), ELISA (Enzyme-Linked Immunosorbent Assays), and immunosensors. Detection in genosensors and immunosensors can be done with electrochemical and/or electrical measurements, as will be further discussed in the last two sections of this article. Even though the techniques mentioned are effective and sensitive, their sensitivity has been influenced by the choice of the principle of detection, viral load, and specific immuno-response of individuals. Indeed, one of the major challenges in the diagnosis of COVID-19 is the need to employ more than one detection strategy owing to the long incubation period of the virus. In addition to detecting genetic material of SARS-CoV-2, which can be performed at any stage, detection of antibodies through serological immunosensing needs to be carried out at different time points after infection. Figure 2 shows a schematic timeline with the most appropriate detection methods along the course of infection.

The profiles for IgG and IgM antibodies of SARS-CoV-2 have been discussed in a few works in the literature.[33,34] They are similar to the ones obtained for SARS-CoV infection, although the time dependences are significantly different.[35,36] For SARS-CoV infections, IgM peaks after 3 weeks from the onset of symptoms, while IgG peaks in the fifth week after onset of symptoms.[35] In the case of SARS-CoV-2, the viral load peaks in approximately 5−7 days after the onset of symptoms. IgG and IgM antibodies have different profiles. The IgM level in the organism peaks within ∼14 days after the onset of symptoms and rapidly decreases in the third week of infection.[34] IgG, however, peaks between the second and third week of infection. Differently from IgM, the IgG level in the infected organism remains high until the fifth week of infection.

In the first days of infection, detection of COVID-19 is mainly performed by quantification of the SARS-CoV-2 viral load. Therefore, genetic-based techniques such as RT-PCR, LAMP, CRISPR, and genosensors are the most indicated (Figure 2, dark blue region, left). The peak of SARS-CoV-2 viral load coincides with the beginning of the immunoresponse to the disease through IgM and IgG production (middle region of Figure 2). In this case, in addition to detection of SARS-CoV-2 genetic material through LAMP, RT-PCR, CRISPR, and genosensors, immunological assays with high sensitivity can be applied for IgG and IgM, including CLIA, ELISA, LFDs, and immunosensors. After the third week of infection, IgG load reaches its maximum in the infected organism as the SARS-CoV-2 viral load remarkably decays. Hence, genetic-based techniques are no longer effective, and detection of SARS-CoV-2 is performed through IgG and IgM quantifications by CLIA, ELISA, LFDs, and electrochemical and electrical immunosensors (Figure 2, soft-blue region, right).

Immunoassays are cost-effective, sensitive, rapid, and selective, but they involve rigorous washing steps which affect automatization.[37] The genetic material-based techniques frequently exhibit improved sensitivity and selectivity in comparison to immunological assays.[37] This is a key advantage for detection of SARS-CoV-2, a virus with high structural similarity to SARS-CoV.[18] Nevertheless, detecting nucleic acids often requires time-consuming analyses and highly skilled operators,[37] being thus disadvantageous for mass testing in a pandemic outbreak. Therefore, there are challenges to be addressed in both types of detection for reaching an effective diagnosis, as will be further discussed in this review paper. Most of the techniques mentioned in this review have already been employed for SARS-CoV and/or MERS-CoV, including RT-PCR,[38] ELISA,[39] LAMP,[40] CLIA,[41] LFDs,[42] immunosensors,[43] and genosensors.[44] Some have been implemented in POC devices for several pathologies, including LFDs,[45,46] CLIA assays,[47] genosensors,[48] electrochemical immunosensors,[43,49] and field-effect transistor devices.[50] Herein, we will discuss important perspectives for adapting these existing technologies for COVID-19 detection.

From the need of different types of diagnostics, one may list the types of materials for the sensing units or test kits, most of which are products of the biotech industry. For detection of genetic material, the kits must contain DNA sequences (primers) which will function as biorecognition elements for different target genes responsible for proteins of the virus, such as ORF1ab, N protein, and S protein, for example. These are the cases of RT-PCR and LAMP assays. The primers are normally immobilized onto different materials depending on the technique and type of assay.[51,52] As usual in any development of a diagnostic tool, the sensing platform is first validated with standard spiked samples before experiments are done with real samples (i.e., swabs, blood, serum, and plasma samples).[3,53] The primers for RT-PCR and LAMP assays are produced by molecular biology methods. In the immunoassays, the biorecognition elements are frequently proteins (biomarkers) which will bind specifically to antibodies immobilized onto the sensing platform.[24,54,55] These devices can be applied to a large number of samples, as blood, serum, plasma, urine, and saliva. There is a large variety of materials onto which these biorecognition elements can be immobilized, including polystyrene in ELISA assays, magnetic beads in CLIA, and nanomaterials in electrochemical and electrical immunosensors. As in the case of genetic-based platforms, immunosensors are validated in spiked samples prior to real sample analyses.[56] The target antibodies and the biorecognition elements are produced by the biotech industry.

■ GENETIC MATERIAL-BASED DETECTION TECHNIQUES

In genetic material-based techniques, SARS-CoV-2 is detected through quantification of its viral RNA. For RT-PCR and LAMP assays, RNA is quantified after its transcription to DNA. CRISPR assays, on the other hand, are based on Case 13 targeting enzyme activity. In this section, we shall discuss the operation principles of these techniques and their application to SARS-CoV-2. Unfortunately, genosensors for SARS-CoV-2 detections have not been developed yet. Because we believe that integrating genosensors in POC devices is a viable route

for mass testing of COVID-19, we shall discuss their earlier use for pathogens such as SARS-CoV.

RT-PCR for SARS-CoV-2 Testing. RT-PCR has been the most used technique for early diagnosis of SARS-CoV-2. RT-PCR, whose scheme to detect mRNA is shown in Figure 3, is already applied to detect several pathogens, including virus and bacteria.[57,58] For COVID-19, this involves detection of the causative virus,[53,59] similarly to its use in other acute respiratory infections. Diagnostics can be made with samples from different parts of the human body, including anal, nasopharyngeal, and oropharyngeal swabs and human fluids such as blood, blood serum, saliva, and urine.[60,61] SARS-CoV-2 could be found in 78% of serum and 50% of plasma samples.[22,62,63] Usually, PCR reactions are applied to DNA amplification and detection by direct reactions with *Taq* polymerase. However, since the genomic material of such viral pathogens as CoVs is RNA, a previous step for viral mRNA conversion to DNA is required. Therefore, the RT-PCR detection mechanism comprises two steps: (1) an RNA-dependent DNA polymerase (reverse-transcriptase) copies mRNA into complementary DNA (cDNA); and (2) *Taq* polymerase is applied to amplify the generated cDNA.[58] Most RT-PCR tests for CoVs are performed with fluorescence measurements and are quantitative, which is why sometimes they are referred to as RT-qPCR. Briefly, cDNA polymerizes with a probe targeted with both fluorescent and quencher labels. After polymerization into double-stranded DNA (ds-DNA), the quencher and fluorescent probes are separated and light emission from the fluorophore is observed upon light excitation.[58]

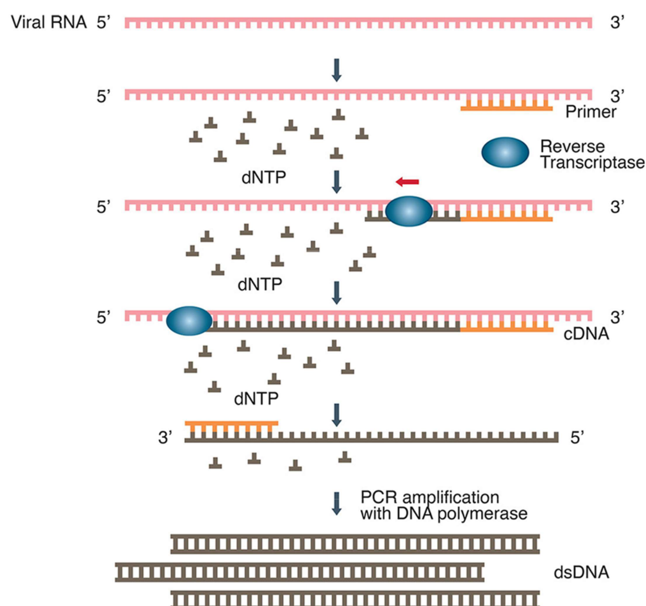

Figure 3. (A) Schematic representation of RT-PCR procedure to detect viral RNA through DNA amplification and detection. Reprinted with permission from ref 70. Copyright 2020, American Chemical Society.

RT-PCR detection is highly reproducible, sensitive, and selective. It is considered the most appropriate method for CoVs detection, including SARS-CoV-2.[58] However, it is time-consuming and expensive, requiring several steps to obtain the viral genome and post-sample treatments in a well-equipped laboratory. These requirements are especially limiting for the needed rapid, low-cost assays for SARS-CoV-2.[58,59,64] Moreover, its reliability for SARS-CoV-2 has been questioned owing to conflicting results depending on the sampling specimens.[65,66] Indeed, false results from RT-PCR analyses were observed for pharyngeal swab specimens from Wuhan hospitals,[67] probably owing to insufficient testing material collected from the patients, laboratory errors, or sample transportation issues.[2] In fact, to improve the reliability of the diagnosis, a recommendation was made to combine RT-PCR results with computer tomography images.[67] Furthermore, variable results from RT-PCR tests involving primers in ORF1ab and N genes were obtained due to variation in RNA sequences.[67] Thus, ORF1ab and N gene were not recommended for RT-PCR testing by institutions throughout the world.[22]

Many efforts have been made to improve RT-PCR in the context of COVID-19 detection. One such example is a high-throughput platform (Cobas 6800) for automated detection, which is helpful to cope with large numbers of samples without any fluctuations in the tests.[68] With this methodology, key steps in SARS-CoV-2 detection were fully automated, including nucleic acids extraction, purification, amplification, and detection. This facilitated sample-handling and data interpretation by people who are not familiar with RT-PCR assays. The samples can be inserted after a rapid processing, reducing the average total time of analysis to less than 30 min (including manual and hands-on steps).[68] Analytical validations regarding inter-run variability, limit of detection (LOD), and cross reactions with other pathogens were performed. For 25 mL of reaction volume, a LOD of 689.3 copies/mL with 95% of detection probability was reached, based on E gene targeting, without false positive results. It should be noted that test validation was made with spiked-in material, and therefore matrix effects from real samples could not be truly investigated.[68] These analytical results were consistent with those obtained by Corman et al., who also used RT-PCR for COVID-19 detection.[53] In this case, however, a workflow was established for situations in which viral genomic material of the virus is absent, which may be an alternative strategy for SARS-CoV-2 testing. The detection mechanisms were based on assays for E and RdRp genes. The latter assay contained specific probes capable of distinguishing SARS-CoV-2 from SARS-CoV, avoiding false positives that could occur due to structural similarities between CoVs. A LOD of 5.2 and 3.8 copies/reaction was obtained for E gene and RdRp, respectively. There was no cross reactivity with other human CoVs, 229E, KHU1, NL63, OC43, and MERS-CoV, showing the specificity of the assays.[53] To reduce the costs of RT-PCR assays, a new protocol was proposed for SARS-CoV-2 negative diagnostics with pharyngeal samples of asymptomatic volunteers with Trizol-RNA purification.[69] Although 3 of 12 volunteers involved in the work had to be retested, the accuracy and sensitivity limit reached with a known lentivirus was of 1−10 virus particles. These results were obtained after a primer validation step for RdRp, N, S and E genes, which is essential for a new RT-PCR assay. The time of analysis was 4 h, and the cost was estimated at less than US$ 15 per sample.[69] Hence, the issues to address for large-scale application of this protocol include the long time for analysis and the inaccuracy for detecting positive cases of SARS-CoV-2.

RT-PCR was also employed as the main diagnostic tool during MERS-CoV and SARS-CoV endemics,[71,72] and there-

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Table 1. Various Types of RT-PCR Protocols for SARS-CoV-2 Detection in the Literature

gene target[a]	positive rate for SARS-CoV-2	LOD (95% of detection probability)	type of samples	ref
RdRp, N, E	55%	Not informed	Faecal samples	74
ORF1ab, NP	40.98% (ORF1ab) and 39.80% (NP)	Not informed	Nasal and pharyngeal swabs, bronchoalveolar lavage fluid, sputum	64
Nsp2 protein	39%	1.8 $TCID_{50}$/mL	Urine, rectal swabs, RTS	75
E-Gene-LDT	Not informed	95.55 copies/mL	Oropharyngeal, nasopharyngeal swabs	3
Not informed	3.33%	Not informed	Tears, conjunctival secretions, sputum	76
E	Not informed	27.6−32.2 (Ct, positive samples)	Oropharyngeal swabs	74
E, S	Not informed (single patient study)	Not informed	Semen, urine	77
RdRp helicase, S, N	28.2% for RdRp helicase, 43.6 for negative RdRd-P2, 24.2% RTS and 8.5% NRTS	11.2 copies/reaction for all genes	RTS, NRTS	78
CD4+, T lymphocytes, CRP, ESR, PCT	16.7% (stool), 6.9% (urine), 21.8 (oropharyngeal, feces)	Not informed	oropharyngeal swab, stool, urine, feces, and serum samples	61

[a]ESR: Erythrocyte sedimentation rate. CRP: C-reactive protein. PCT: ProcalcitoninCD4+: Cluster of differentiation 4. NP: nucleocapsid protein. RTS: Respiratory tract specimens. NRTS: nonrespiratory tract specimens. $TCID_{50}$: 50% tissue culture infective dose. Ct: Threshold cycle.

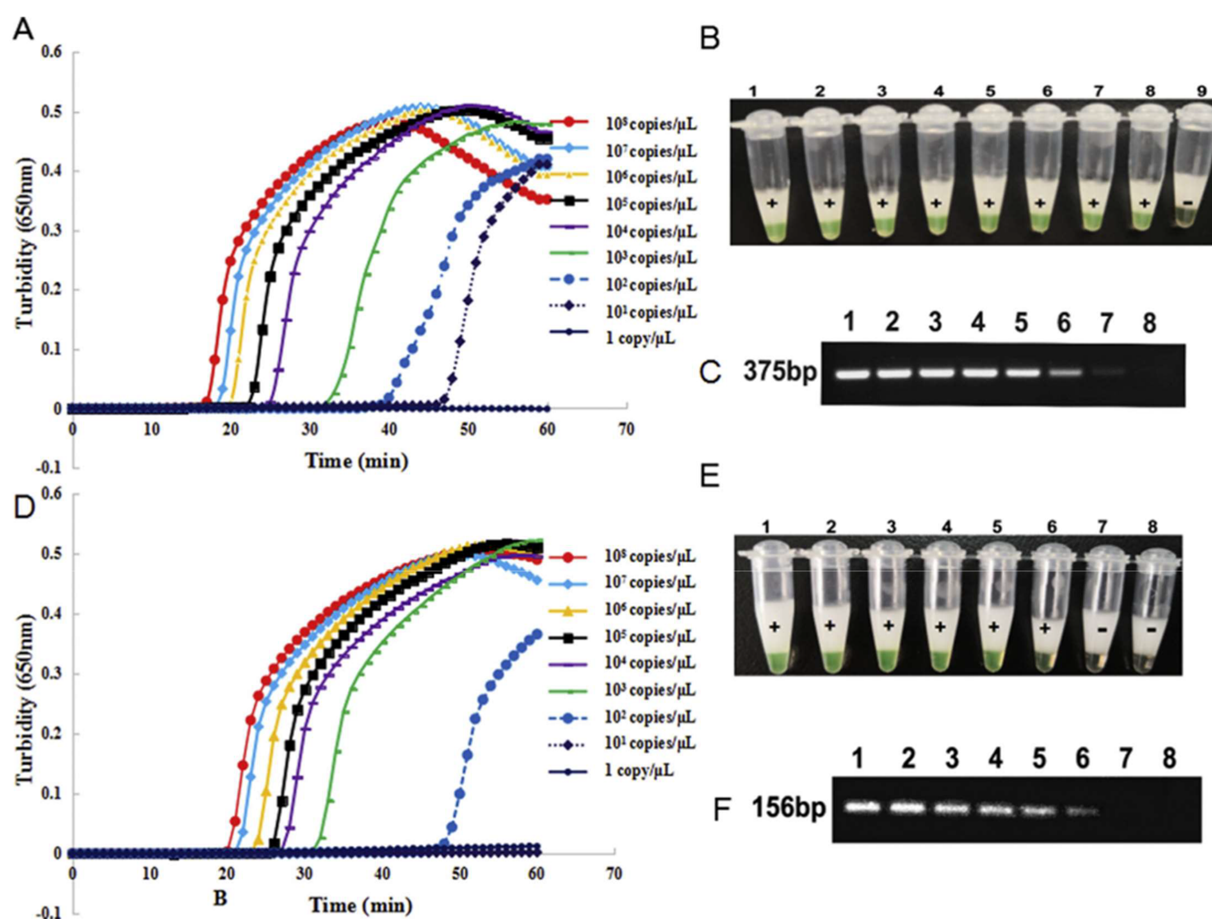

Figure 4. Sensitivity susceptibility to the primer gene for LAMP analyses. (A,B) RT-LAMP sensitivity toward ORF1ab gene targeting for SARS-CoV-2 detection based on ORF1ab-4 primer; (D,E) sensitivity of the proposed RT-PCR assay for SARS-CoV-2 S protein targeting using primer set S-123; (C,F) Conventional PCR assay sensitivity concerning both ORF1ab and S genes targeting for SARS-CoV-2 detection. Reprinted with permission from ref 81. Copyright 2020, Elsevier Publisher.

fore was successfully adapted to SARS-CoV-2. For example, the protocol for MERS-CoV detection based on N gene targeting may complement other gene targeting,[73] such as upstream MERS-CoV E gene (upE).[22,73] A remarkable specificity was achieved, which was confirmed by the lack of false positive amplifications with other human CoVs. Although satisfactory analytical results were obtained, this study was limited by the low amount of available real samples, and therefore the latter data reflect mock specimens spiked with the virus. Furthermore, storage conditions, handling, and collection for all the spiked samples were not patterned, leading to the lack of reliability of the results.[73] Hence, a stricter sample preparation protocol must be adopted if one decides to apply these assays for SARS-CoV-2 detection. In fact, important parameters optimized for other CoVs cannot be reproduced for a successful analysis of SARS-CoV-2

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infections.[22] On the other hand, interference from other pathogens, e.g., other CoVs, may appear in case of simultaneous presence of these viruses in the sample. For instance, peaks of viral load for SARS-CoV, MERS-CoV, and SARS-CoV-2 are different. SARS-CoV and MERS-CoV viral load peaks were observed during the second week of infection, while the SARS-CoV-2 viral peak occurs in the first week of infection. Another important aspect concerns sampling specimens for RT-PCR analyses of viral RNA, which cannot be the same for the latter CoVs: SARS-CoV and MERS-CoV presented a positive rate of infection of approximately 100% for LRT (lower respiratory tract) specimens. SARS-CoV-2, in its turn, was more detectable (higher positive rate for RNA) with URT (upper respiratory tract) specimens.[22] This suggested that CoVs have different viral kinetics depending on the sampling specimens, and therefore, this is a new parameter to be optimized in RT-PCR. Nucleic acids testing by RT-PCR in blood samples has been effective to monitor SARS-CoV and MERS-CoV,[63,72] with the high viral load associated with the severe disease stage in the latter endemics.

Table 1 lists some RT-PCR assays for SARS-CoV-2 detection with figures of merit (as LOD), or from initial clinical studies. In this table, the positive rate found for SARS-CoV-2 with each assay was also reported. The positive rate should be interpreted as the rate of effective viral RNA detection with the respective RT-PCR assay and the chosen sample specimen.

Loop-Mediated Isothermal Amplification (LAMP) Assays. LAMP is a recent nucleic acid amplification technique, alternative to RT-PCR, which is based on a set of four designed primers and a strand displacement of active DNA polymerase. It is able to produce DNA targets (up to 10^9) in less than one hour under isothermal conditions (~63 °C).[79,80] LAMP assays have high specificity, sensitivity, simplicity of operation, and short time of analyses,[79] being more sensitive, stable, and resistant to inhibitors than PCR assays. This allows application with minimal sample preparation and less extensive nucleic acid extraction.[79] Because it is performed at a fixed temperature, its application in a POC assay for SARS-CoV-2 detection has been recommended.[58]

Similar to RT-PCR, RT-LAMP employs RNA transcription into DNA with DNA polymerase, but the selectivity is higher owing to the larger number of primers. RT-LAMP was applied to MERS-CoV with selectivity in the presence of similar CoVs,[40] and has been adapted to the diagnosis of COVID-19. In-house and commercial RT-LAMP assays reported for SARS-CoV-2 are based on primers for different genes, such as ORF1ab,[80−82] E protein,[80] S protein,[81−83] RdRp,[82] Nsp3,[83] ORF8,[83] ORF1a,[84] and N protein.[82−84]

To provide quick diagnosis, colorimetric detections were adopted.[83,84] RT-LAMP has been much less used than RT-PCR, but its sensitivity and reliability have been comparable to those of RT-PCR[80,82,84] In the study by Park et al., however, the LOD (100 copies per reaction) was not sufficient to detect COVID-19 in infected patients, which was attributed to the inadequate choice of target sequences based on SARS-CoV criteria.[83] Therefore, the correct choice of primers to be used in RT-LAMP assays seems to interfere not only on selectivity of the assay, but also on its ability to detect SARS-CoV-2 genetic material at trace levels. Yan and co-workers demonstrated that the accuracy may depend on SARS-CoV-2 mutations on the primer sequence of the target gene, as shown in Figure 4.[81] In fact, this limitation is also present in other genetic material-based assays, including RT-PCR.[85] Since RT-LAMP depends on using different primers and only shows optimum results at high temperatures, its application is limited and quite challenging under ordinary conditions. Besides, LAMP assays are not as useful as RT-PCR for mass testing because of the need of sophisticated (and, thus, expensive) thermal cycling equipment.[84] Another challenge faced by scaling-up RT-LAMP assays regards the need of genetic primers for the nucleic acid amplification reactions, which are expensive and nontrivial reagents to be obtained and cannot be stocked for a long time.

CRISPR-Based Biosensing Strategies. CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated) is a powerful analytical tool to modify genomes and gene functions.[86−88] A recent discovery of Cas13 as an RNA targeting enzyme in CRISPR-Cas has sparked excitement for next-generation biosensors with higher specificity, sensitivity, and rapid detection of nucleic acids.[86] Cas13 is an RNA-guided RNase capable of producing multiple cleavages in the nonspecific target single-strand RNA (ss-RNA).[89] To make it target specific, a CRISPR RNA (crRNA) is needed, and the resulting Cas13-crRNA complex has a higher target specificity for being guided to a RNA sequence of interest.[90] Thus, RNA sensing of Cas13 with nonspecific endonuclease activity is used as a detection strategy, where this enzyme modified with an RNA guide sequence is activated after combining with the specific target.

A fluorescent signal is produced as the activated enzyme interacts with a fluorophore quencher, which indicates the presence of RNA or DNA with high sensitivity and selectivity, up to fmol L^{-1}.[91] The collateral activity of the CRISPR Case family, particularly Cas13, is a triggered cleavage process with self-amplification ability, thus making the system highly sensitive and selective to detect nucleic acids.[88] In addition to the fluorescently labeled based detection, other strategies include visual detection of liquid−liquid phase separation in turbid solutions and lateral flow detection with antigen-labeled reporters.[92,93] The CRISPR/Cas13 biosensing technology has been used to detect ss-RNAs level of Zika virus,[94] dengue virus,[95] and micro-RNA (mi-RNA) in serum samples of brain-tumor patients.[96] In the latter investigation, an integrated low-cost, portable CRISPR/Cas13a biosensor was used to measure the electrochemical signal from miR-19b (brain tumor marker) with detection limit of 10 pmol L^{-1} within a total processing time of less than 4 h.[96] The technique can therefore be fast and low-cost, and does not require sophisticated laboratory equipment.

The sensitivity of the CRISPR/Cas13 method can be enhanced by introducing an isothermal preamplification step in a platform referred to as SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing).[97] This system uses recombinase polymerase amplification (RPA) of RNA or DNA and depends upon the introduction of T7 promotor (RNA polymerase), enabling RNA transcription followed by detection with Cas13. A protocol using SHERLOCK technology has been published to detect target RNA sequences with synthetic S and ORF1ab genes of SARS-CoV-2.[98] RNA sequences at concentrations as low as 20 amol L^{-1} were detected using lateral flow detection readout. The CRISPR/Cas13a assay has been reported with faster analyses than RT-PCR.[99] It can also be used as a benchmark for further advancements to provide a rapid, easy-to-handle, and sensitive system for mass testing.

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One of the most prominent challenges for the application of CRISPR-based strategies is the noticeably high complexity of the technique. It requires an expert team and nontrivial reagents. Other difficulties include lack of standardization of tests, sequence limitations, and limited literature in CRISPR assays. Moreover, an additional step of DNA amplification is often needed to lower the LOD.[100] Owing to these characteristics, CRISPR technologies are less economically viable than RT-PCR and LAMP assays.

Genosensors. Genosensors are a useful, cost-effective alternative to RT-PCR for detecting viral RNA fragments of specific sequences. Similar to RT-PCR, genosensing is based on nucleic acid testing via hybridization between a probe, usually a single strand oligonucleotide, and a complementary DNA or RNA.[101] Upon hybridization of the probe immobilized on the sensor surface, a fluorescent, electrical, or electrochemical signal is produced, thus allowing the biorecognition of the target RNA. In contrast to RT-PCR, the genosensing approach is free from amplification and separation steps, making it simple and easy to handle. It has been used in food analysis[102] and environmental control,[103] but only recently in POC diagnosis.[101,104,105] Indeed, there is a demand for further developments of commercial products for mass testing, which may explain why genosensors for SARS-CoV-2 have not been reported yet. Because genosensors usually involve immobilization of specific probes for hybridization, these devices are of significant selectivity and rarely affected by interferents. This is relevant for the detection of SARS-CoV-2 in the presence of other CoVs, for example.

The prospects of employing genosensing assays for COVID-19 diagnosis are nevertheless promising because related technologies can be adapted. For instance, a genosensor device with a sandwich assay detected the Zika virus in samples of infected individuals, including saliva, serum, and urine in the presence of 1000-fold higher concentrations of dengue and chikungunya homologues.[106] A miniaturized gold-based genosensor platform detected SARS-CoV with an electrochemical technique with an LOD of 6 pmol L^{-1}.[44] The genosensor was sufficiently selective to distinguish 2-base and 1-base mismatches between complementary and unpaired hybrids.[44,107] Both genosensors detailed in refs 44 and 107 contained biotin-labeled probes. For the SARS-CoV genosensor, optimized performance was reached with a judicious choice of matrices and approaches to immobilize the complementary strand to a 30-mer sequence that encodes specific SARS-CoV regions.[44] It is in this aspect that materials chemistry and materials science may play a crucial role, since behind the simplicity of the genosensing methodology is the background knowledge acquired over decades of combining nanomaterials and biomolecules for sensing and biosensing (for a detailed review, see ref 108).[108] Many are the examples of successful results with genosensors depending on leveraging nanomaterials for matrices and principles of detection.[109] In the detection of a cancer biomarker, for instance, the organization of self-assembled monolayers was crucial to allow for distinguishing cell lines of neck and head cancer.[110] For SARS-CoV-2 genetic material detection, a supersandwich-type genosensing device was developed for early detection of viral RNA without amplification steps. The LOD reached of 200 copies/mL was claimed as the lowest in the literature up to the present day.[111] The detection assay allows the monitoring of the results through a smartphone. The development of these types of genosensing devices allow diagnosis with high sensitivity, which is suitable for mass-testing. On the other hand, the extensive preparation steps of the electrode and long incubation times can be considered time-consuming.

Therefore, this dependence on genosensor constituent materials should be considered for adapting current methodologies for COVID-19 diagnosis. While this dependence also applies to immunosensors, as discussed in the next section, the demand for rapid testing with genetic materials is a more urgent one while the COVID-19 pandemic lasts. On the other hand, despite its great selectivity, sensitivity, and other advantages, the scaling-up of genosensing technologies for quick mass COVID-19 technologies may face a number of challenges. As an example, the preparation of genosensing devices usually requires a long time and expensive reagents that cannot be stored at room temperature. Moreover, genosensing devices also demand non-ordinary transport conditions and normally present short shelf-lives.

In summary, the genetic materials-based diagnostics are rapid, sensitive, and specific and can enable the quantitative detection of SARS-CoV-2 RNA and hence early and large-scale detection of SARS-CoV-2. Nevertheless, their testing procedures are complex, in addition to their high cost and slow delivery to the end users. For example, building a RT-PCR processing lab may cost up to 15,000 USD to produce RT-PCR kits of 100 USD each,[1] in addition to the high ratio of false positive and false negative results, which occurs most routinely in these diagnostics tests.

■ IMMUNO-BASED TECHNIQUES

In this section, operation principles and the applicability of immunologically based techniques, such as ELISA, CLIA, and Lateral Flow assays, will be described in the SARS-CoV-2 detection scenario. We will first discuss the use of POC assays for COVID-19 diagnosis, and highlight some of the challenges inherent in these assays. Some important aspects of immunology toward SARS-CoV-2 proteins will be discussed. As we did for genetic material-based techniques, perspectives will also be presented of the use of nanomaterials to improve performance. Emerging immunosensors for SARS-CoV-2 detections will be mentioned, in addition to existing immunosensors developed for other CoVs.

Point-of-care (POC) immunodevices provide a useful platform for SARS-CoV-2 detection. Most POC devices have used immunological testing mechanisms, since tests of nucleic acids (such as viral RNA, for example) in these platforms are not straightforward.[112] It should be noted, however, that electrochemical and impedimetric genosensors are now also considered for POC purposes[48,113] as we discussed previously. POCs have been used for real-time identification of CoVs and other pathogenic diseases,[114] including SARS-CoV and MERS-CoV.[42,115,116] A successful detection of CoVs requires a suitable target protein and its corresponding antibody. For SARS-CoV-2, the most studied protein is the S protein with 24.5% non-conserved amino acid sequence of SARS-CoV. Distinguishing between SARS-CoV and SARS-CoV-2 was possible owing to these divergences in amino acid sequences, especially the antigenicity differences in RBD attributed to the low similarity in the non-conserved amino acid sequences.[117] SARS-CoV-2 S protein is not inhibited by polyclonal anti-SARS-CoV S1 antibodies T62.[117,118] Despite the differences in amino acid sequences, cross-reactivity between SARS-CoV, MERS-CoV, and SARS-CoV-2 S proteins antibodies is still

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possible in immunoassays. Indeed, SARS-CoV specific antibodies, such as the CR3022 human monoclonal antibody, bind to SARS-CoV-2 RBDs.[119] Furthermore, polyclonal goat antibodies anti-MHV S AO4 could be used to detect three CoVs S proteins. This suggested the existence of a conserved epitope in SARS-CoV, MERS-CoVs, and SARS-CoV-2.[118] The selective detection of these viruses using goat anti-MHV S antibodies AO4 by POC assays is not possible. Consequently, to avoid cross-reactivity and improve selectivity for COVID-19 diagnosis, specific recombinant antigens are needed. Therefore, some of the immunoassays for other CoVs based on S proteins should be carefully reviewed and readapted for SARS-CoV-2 diagnosis. An alternative protein that can be used for SARS-CoV-2 detections is N protein. There is evidence that SARS-CoV-2 may be detected through its N protein target alone or combined to S protein in different POCs.[1,120,121] Detection through N proteins already proved to be effective in immunoassays for MERS-CoV and SARS-CoV.[116,122]

Large-scale COVID-19 diagnosis in real blood samples has been made by targeting S protein with IgG and IgM antibodies in POC assays.[5] IgM and IgG sero conversion occurs simultaneously or sequentially in SARS-CoV-2 infected humans.[33] IgG reaches its maximum level in human blood after 17−19 days from the onset of symptoms, while IgM reaches its peak within 20−22 days after this onset.[33] IgM and IgG activity involves SARS-CoV-2 S and N proteins,[123] as confirmed by measuring their binding kinetics to S and N proteins.[124] Most intensive care unit (ICU) patients had higher concentrations of S and/or N IgG antibodies, probably due to the longer-term viral infection. Detection of N protein-based IgM and IgG was more efficient for early identification of the infection, owing to the immunogenicity and intracellular accumulation before virus packaging (i.e., virus assembly).[124] Some of these tests were less sensitive than S protein counterparts.[121] Another important aspect of N and S proteins regards their cross reactivity. Some degree of cross reactivity of SARS-CoV N and S protein was observed for positive COVID-19 serum samples regarding IgM and IgG responses.[33] A combined detection of N and S proteins by their IgM and IgG appears to increase the SARS-CoV-2 detection rate in early infections (up to 75% of patients).[33,124] Another antibody employed for SARS-CoV-2 detections in POC immunoassays is IgA, which can target the S protein RBD in immunoassays.[125,126] Its kinetic response in COVID-19 patients, however, remains unknown.[126] The concentration of IgA peaks during the third week of infection, being more persistent than IgM.[127] Therefore, different POC assays exist for CoVs immunological testing (including SARS-CoV-2) with IgM, IgG, and IgA antigen−antibody interactions.[125−130]

In contrast to the genetic material-based techniques, the immunosensing diagnostic tools could provide cost-effective diagnosis at primary health care units. Except for a few of these methods, such as ELISA which requires large readout devices, anticoagulants, and trained personnel, the majority of them do not need expensive laboratory infrastructure and a lot of reagents.[131] Therefore, they can be easily implemented outside a laboratory. Although we could not find the price of single immunosensing devices in the literature, they are considered less expensive due to their simple assay protocols. These protocols include three or four steps: sample injection, reaction, visualization/interpretation of the results. Therefore, they are economically much more viable than molecular or nucleic acids-based techniques.

Enzyme-Linked Immunosorbent Assays (ELISA). ELISA assays are popular immunoassays[132] to detect pathogens via quantification of antibodies[133] made to interact with antigens adsorbed on a solid support, e.g., polystyrene.[133] In an ELISA assay, the sample is first incubated in a polystyrene tube coated with the antigen. Then, enzyme-labeled Ig antibodies are added to the tube. After antigen−antibody interactions and washing steps, the remaining enzyme provides a quantification of the specific antibody in the serum sample. Antibodies can also be detected by other similar ELISA strategies, in three different assays (competitive, inhibition, and double-antibody).[133]

ELISA assays for SARS-CoV-2 have been developed for direct (sandwich) or indirect IgG and IgM detection,[121,134−136] this latter also called as capture or sandwich approach. Figure 5 shows a detailed comparison of these two methodologies.[57] Competitive ELISA is based upon the competitive binding of the sample analyte and a reference to the limited quantity of the adsorbed antibody.[133] This latter approach is only adopted for small peptides containing a single recognizable binding epitope. For example, Liu and co-workers observed that ELISA assays for IgM detection of S protein of SARS-CoV-2 are more sensitive than for the N proteins.[134] The sensitivity in IgG detection, on the other hand, was similar for S and N proteins. Therefore, the assays can be employed as a complementary test to RT-PCR, particularly for serum samples of COVID-19 patients after 10 days post-disease onset.[134] The main advantages of ELISA assays, especially for COVID-19 diagnosis, are the high sensitivity, uniform testing for a wide range of patients, speed, and simplicity of operation.[136] However, when direct antibody screening is employed to improve simplicity and rapidness of analyses, false positive results may be recorded. This is frequently related to interference factors. As an example, Wang and co-workers stated that interference in ELISA assays for IgM detections occurs.[137] It was found that rheumatoid factors at mid-to-high levels can lead to false positive IgM results. These data were obtained through urea dissociation tests.[137] Indirect antigen or

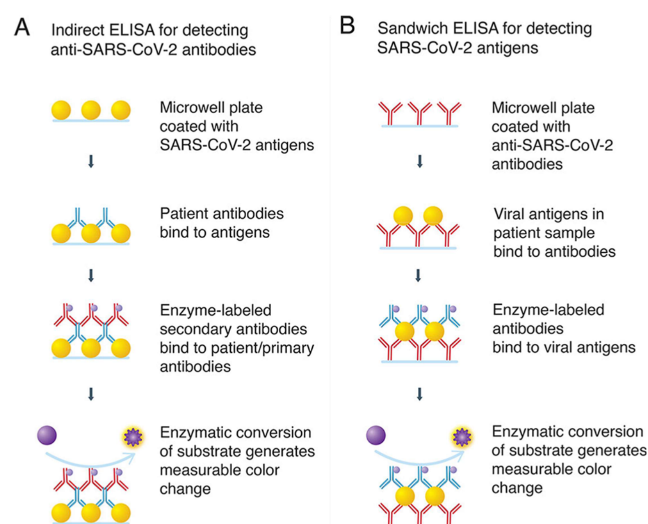

Figure 5. Brief description of operation modes of both (A) sandwich and (B) indirect ELISA assays for detecting SARS-CoV-2 antigens. Reprinted with permission from ref 70. Copyright 2020, American Chemical Society.

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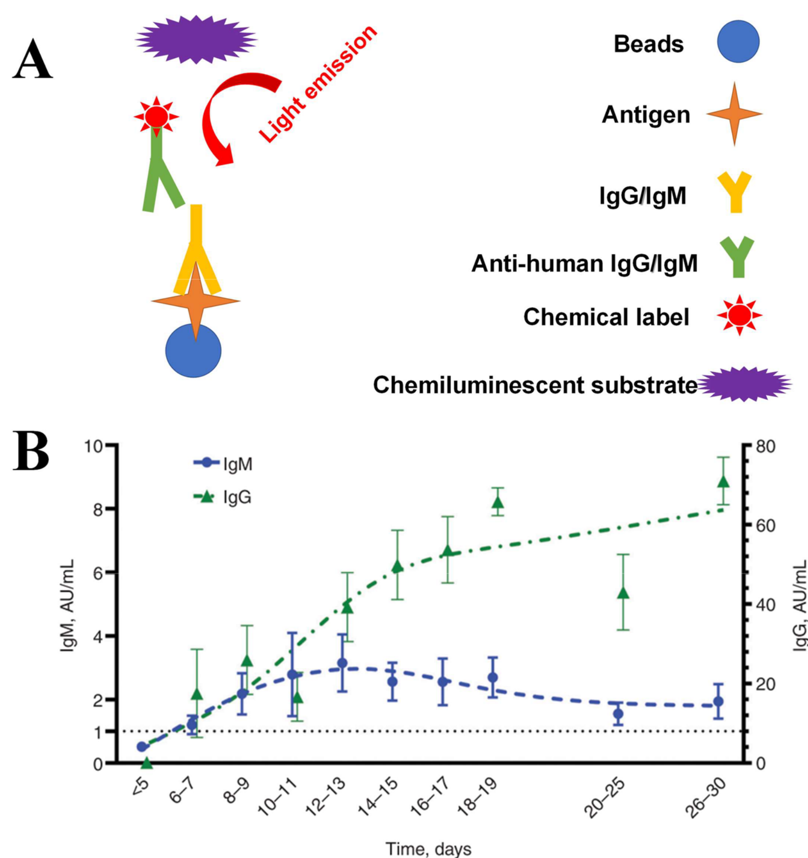

Figure 6. (A) Schematic representation of operation principles of CLIA assays. (B) IgG and IgM antibody quantification through CLIA assays versus days of infection by SARS-CoV-2. Reprinted with permission from ref 140. Copyright 2020, Walter de Gruyter GmBH &Co. KG.

Table 2. Types of Commercial and Developed ELISA and CLIA Immunoassays Based on IgG and IgM Antigenic Activity Towards SARS-CoV-2 Proteins

immunoassay/ test name	target	antibody	sensitivity	LOD	cutoff	ref
ELISA	SARS-CoV-2 S1 subunit of S protein	IgG/IgA	Not informed	Not informed	1.1 kAU/L (IgG, IgA)	127
ELISA	S and N recombinant SARS-CoV-2 proteins and HRP-conjugated antigen	Ab/IgG/ IgM	89.6–100 (Ab), 54.1–79.8% (IgG), 73.3–94.3% (IgM)	Not informed	Not informed	152
rN and rS based ELISA	SARS-CoV-2 recombinant S and N proteins	IgG/ IgM	74.3% (IgG), 77.1% (IgM)	Not informed	Not informed	134
ELISA HB300E analyzer	SARS-CoV-2 S and N proteins	IgG/ IgM	33.3%	Not informed	Not informed	153
ELISA Euroimmun	Recombinant S1 structural SARS-CoV-2 protein	IgG/IgA	84%	Not informed	Not informed	125
CLIA Maglumi	SARS-CoV-2 recombinant antigen labeled with ABEI	IgG/ IgM	64.3%	Not informed	Not informed	125
CLIA iFlash 1800 Analyzer	N and S SARS-CoV-2 proteins	IgG/ IgM	73.3% (IgM); 83.3% (IgG)	Not informed	7.1 AU/mL (IgG); 10 AU/mL (IgM)	120
CLIA iFlash 300 analyzer	N and S SARS-CoV-2 proteins	IgG/ IgM	Not informed	Not informed	Not informed	154
CLIA Axceed 260 analyzer	N and S SARS-CoV-2 proteins	IgG/ IgM	Not informed	Not informed	Not informed	155

antibody screening, on the other hand, may be affected by nonspecific immobilizations.[138] Besides these challenges, cross-reactivity between SARS-CoV-2 and SARS-CoV can also happen, specifically with the EUROIMMUN ELISA assay for IgG detections.[139] Interference from other matrix components, such as hemoglobin, triglycerides, and bilirubin, was also evaluated, with no significant interference on the assay result.[139]

Chemiluminescent Assays (CLIA). Strategies using antibodies include chemiluminescent immunoassays (CLIA) described in Figure 6A.[140] CLIA are attractive due to possible automation, high specificity, low level of interferences, high stability of conjugates and reagents, reduced incubation time, compatibility with immunological assays protocols, and wide dynamic range.[141] Validation of CLIA assays is frequently performed by comparing with results from ELISA. The large-scale application of CLIA is hampered, however, owing to

limited test panels, high costs, and nonlinear response to the analyte concentration due to complex chemical reactions.[138] In CLIA assays, a luminescent label acts as indicator of the chemical reaction in a direct or indirect methodology. For direct methods, acridinium and ruthenium esters are luminophores, while enzymatic markers (alkaline phosphatases, horseradish peroxidase with luminol, and AMPPD) are applied for indirect methods.[138] Antibodies may be labeled with different types of materials, including nanomaterials that possess increased surface electron density and surface area. This leads to signal amplification,[56,142] as illustrated with the use of gold nanoparticles,[56,143] magnetite nanoparticles,[56,144] and Au−Ag alloy nanoparticles.[145]

As occurs with ELISA assays, hemoglobin, triglycerides, bilirubin, and rheumatoid factor are matrix compounds which can interfere on CLIA results.[146] In addition, because CLIA is an immunoassay, other compounds such as autoantibodies, endogenous compounds, and human anti-animal antibodies can also interfere and lead to false results. A detailed review on interference in imunoassays is given in ref 147.

Detection of SARS-CoV-2 using CLIA has been mostly performed by targeting N and S proteins through IgM and IgG antibodies.[121] CLIA commercial assays had comparable sensitivity with ELISA and LFDs (lateral flow devices) POC devices,[125,148] but less specificity.[121] Montesinos and coworkers compared two automated assays for detecting SARS-CoV-2 in serum samples. Euroimmun IgG/IgA ELISA assays were more sensitive than IgG/IgM Maglumi CLIA assays,[125] consistent with results for other pathogens.[149] The results from a commercial CLIA assay for IgM and IgG are shown in Figure 6B, where the concentration of each antibody was monitored along the course of the infection, starting from the fever onset.[140] Deviations in the results were less than 4% and 6% for IgM and IgG, respectively. The detection of SARS-CoV-2 through fully automated CLIA assays has been reported.[150] The mass application of these automated assays is a challenge for low-budget locations, and there is the possibility of false negative results, especially at early-stage of infections.[150] Therefore, other methodologies are required for the correct management and treatment of patients. Table 2 lists some ELISA and CLIA assays used for SARS-CoV-2 detection. Most of the articles do not present a LOD value or even the obtained cutoff for the studied assay. These latter parameters are essential to evaluate the minimum amount of analyte that can be detected by the assay. This issue is frequently related to misinterpretation of the meaning of sensitivity in the medical area. A thorough discussion of this topic is provided in ref 151.

Lateral Flow Devices (LFDs) for Point-of-Care Testing. POC immunoassays in LFDs provide quick, efficient, and inexpensive testing through immunochromatographic mechanisms. LFDs are paper-based strip devices comprising four main regions: (1) cellulose-based sample pad for sample dropping; (2) conjugate pad, generally made of glass fiber containing the labeled particles (generally colorimetric or fluorescent) conjugated to the analyte receptor; (3) nitrocellulose-based detection pad with test lines, and control lines—test lines contain biomolecules capable of binding to the analyte-label-conjugated particles (if the target analyte is present in the sample); a signal proportional to the analyte concentration is generated, while control lines are used to verify the test operation, capturing labeled-conjugated particles independently of the presence of the analyte; (4) absorption pad, also made of cellulose, which helps induce sample flow through the test strip.[156] The limitations of POCs with LFDs are related to the low sensitivity toward the target analyte and cross-reactivity with other species in the sample matrix. Moreover, the viscosity of the liquid samples needs to be within a certain range, which prevents a wider use of such devices.[156] Interference from matrix blood samples, as discussed in CLIA and ELISA assays, should also be taken into account for LFDs immunoassays. A number of other difficulties may exist for application of LFDs, which include denaturing of immobilized proteins (i.e., antigens), nonspecific adsorption, nanoparticle aggregation, and steric hindrance.[157] The performance of the assays may also depend on the paper used in some LFDs. To exemplify, trapping of biomolecules on paper pores results not only in binding undesired effects, but also in flux rate changes.[157] To cope with these problems, several pretreatments of the pads are carried out.[158] An illustration of a LFD device likely to be used for SARS-CoV-2 is depicted in Figure 7.

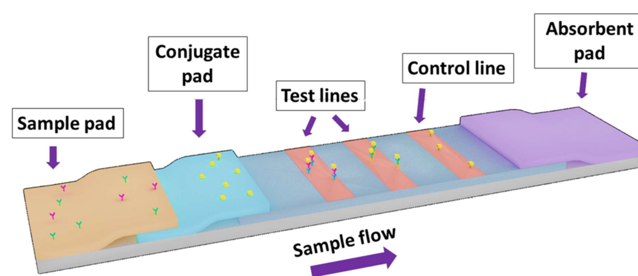

Figure 7. Representation of a typical LFD assay that can be employed for SARS-CoV-2 testing. The design of commercial assays is the same as that represented above. The device comprises a sample pad in which IgM and IgG antibodies are immobilized. The conjugate pad contains gold nanoparticles (AuNP) conjugated to a SARS-CoV-2 antigen. In test lines, anti-human IgG and anti-human IgM are immobilized to interact with IgG-AuNP-antigen and IgM-AuNP-antigen complex. The control line contains nonhuman reactive anti-IgG or anti-IgM. The absorbent pad is useful for maintaining sample flow through the strip.

In spite of these limitations and challenges, LFDs have been applied for COVID-19 diagnosis in human blood and serum samples,[1,4,130,135] in most cases to confirm suspected and asymptomatic cases.[4] In comparison to ELISA and CLIA assays, LFDs diagnostics shows satisfactory sensitivity and accuracy for SARS-CoV-2 detection.[125] The sensitivity with nine commercially available immunochromatographic gold-labeled LFDs for IgM and IgG detections ranged 72.7−100%, with results obtained in less than 20 min.[159] Since LFDs are not quantitative for COVID-19, the sensitivity parameter adopted is different from those used in analytical chemistry. This difference was clarified by Lassaunière et al., who defined sensitivity as "the number of patients which were correctly diagnosed with SARS-CoV-2 infections by the studied POCs after a previous positive SARS-CoV-2 diagnosis by nucleic acids testing with respiratory samples".[130] Most LFD tests for SARS-CoV-2 are based on S or N proteins as antigens, but a large number of commercial LFDs assays do not specify the virus protein chosen as antigen. Although Whitman and collaborators reported the maximum sensitivity in their LFDs detections for COVID-19 diagnosis, they focused on the report of the "positivity" rate obtained, which increased significantly after 2 weeks of disease conditions.[160]

Table 3. Commercial and Recent LFDs for COVID-19 Diagnosis[a]

immunoassay/test name	target	SARS-CoV-2 species	sample	sensitivity	ref
LFD Avioq	IgG/IgM	Recombinant SARS-CoV-2 antigen	Human serum	68.8%	125
LFD/LNPs labeling	IgG	SARS-CoV-2 N protein	Human serum	Not informed	163
LFD	IgG/IgM	RBD of S protein	Blood samples	88.66% (positive results); 90.63% (negative results)	128
LFD	Ab/IgG/IgM	SARS-CoV-2 N and S proteins	Plasma samples	97.5% (Ab); 86.3% (IgG) 88.8% (IgM)	153
LFD AutoBioDiagnostics	IgG/IgM	Not informed	Human serum	93%	130
LFD DynamikerBiotechnology	IgG/IgM	Not informed	Human serum	90%	130
LFD CTK Biotech	IgG/IgM	Not informed	Human serum	90%	130
LFD ArtronLaboratories	IgG/IgM	Not informed	Human serum	83%	130
LFDs (DeepBlue, Bioperfectus, UCP)	IgG/IgM	Not informed	Human serum and plasma samples	84.3−100%	160

[a]LNPs: Lanthanide-doped polystyrene nanoparticles. Ab: Total antibody. RBD: Receptor binding domain.

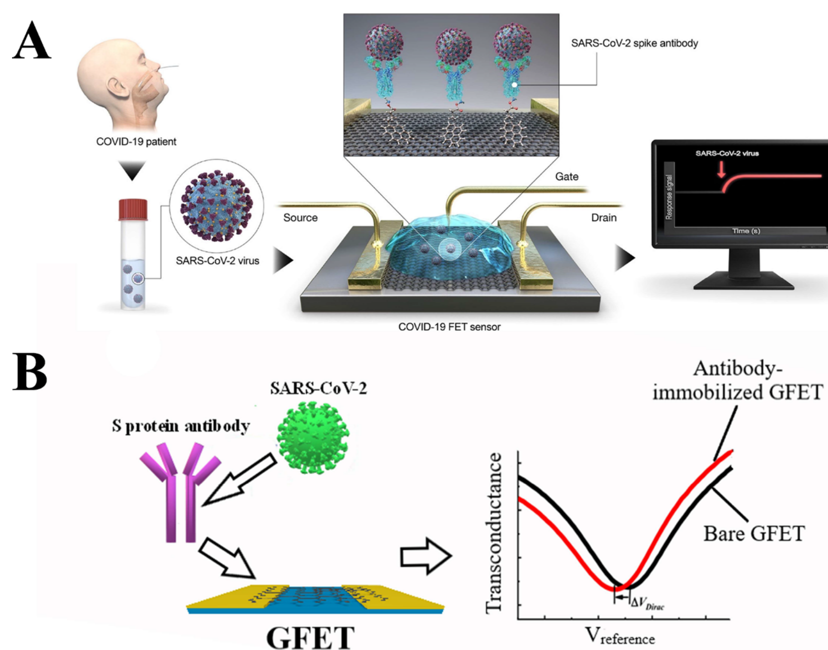

Figure 8. (A) Application of GFET-based electrical immunosensor with SARS-CoV-2 S protein antibodies immobilized onto graphene surface. The device was employed for SARS-CoV-2 detection in clinical samples from COVID-19 infected patients. Reprinted with permission from ref 182. Copyright, 2020, American Chemical Society. (B) Schematic representation of GFET immunosensor for RBD of S1 subunit. V_{ref} shift due to antibodies immobilization in comparison to the bare graphene surface.

Diagnosis of SARS-CoV-2 with LFDs has also benefited from recent advances in incorporation of nanoparticles.[45,161,162] For instance, lanthanide-doped polystyrene nanoparticles were employed instead of conventional AuNP as fluorescent labels for detecting IgG through N protein immobilization.[163] The assay was reproducible (coefficient of variation <15%) with detection results comparable to RT-PCR, showing the suitability for detection of suspicious and asymptomatic COVID-19 cases.[163] However, in contrast to RT-PCR assays, LFDs assays are limited at the onset of infection, because production of IgG and IgM antibodies has not yet begun.

Colloidal gold nanoparticles were studied by Huang and co-workers on a simple and easy-to-handle LFD platform to detect SARS-CoV-2 N protein using an anti-human IgM−colloidal gold nanoparticle conjugate. Despite the similarity between these assays and the commercial LFDs for SARS-CoV-2 detection, selectivity and sensitivity achieved were satisfactory and up to 90% in both cases.[164] However, this research is at an early stage of development. Indeed, there is no report of a well-established methodology using these innovative LFDs for COVID-19 diagnosis. This highlights another important challenge for the use of LFDs: the successful establishment of novel nanobiointerfaces for the development of new LFDs within an acceptable time frame. A possible alternative is to employ nanomaterials for antibody conjugates and colorimetric responses on test strips, which could be viable for COVID-19 diagnosis.

Some of LFDs for SARS-CoV-2 immunodetection are summarized in Table 3.

Electrochemical and Field-Effect Transistor-Based Immunosensors as Point-of-Care Devices. Electrochemical and electrical immunosensors have been incorporated into POC devices for many pathogens and analytes,[165−170] particularly with incorporation of nanomaterials (including 2D materials).[171−178] Electrochemical immunosensors have

indeed been used for MERS-CoV,[43] but surprisingly, we have been able to find only one example for SARS-CoV-2, with the eCoVSens platform. A fluorine-doped indium−tin oxide electrode was modified with gold nanoparticles and COVID-19 monoclonal antibodies for detecting the S1 domain of S protein.[179] Spiked samples were analyzed within 10−30 s, and the LODs of 90 and 120 fmol L^{-1} were reached.

Among the immunosensors containing nanomaterials, of special relevance are the graphene field-effect transistors (GFETs) in which biosensing exploits changes in the electrical properties of the interface induced by analyte approximation and/or adsorption.[172,180,181] High performance is expected owing to the high basal plane conductivity of graphene, and its dependence on surface changes.[173,182] The first GFET developed to detect SARS-CoV-2 is illustrated in Figure 8A.[182] Antibodies for the S protein were immobilized through 1-pyrenebutanoic acid and succinimidyl ester probe linkers. Detection was made by measuring the drain current with clinical samples prepared from nasopharyngeal swabs, under a constant bias of 10 mV.[182] The device was capable of distinguishing infected from noninfected samples, with a detection limit of 0.42×10^2 copies/mL.[182] The LODs for other samples tested were 1 fg/mL, 100 fg/mL, and 16 pfu/mL in phosphate buffer saline, clinical transport medium, and culture medium, respectively.[182] Another GFET immunosensor applied to SARS-CoV-2[24] had antibodies from SARS-CoV S1 subunit immobilized onto graphene, as illustrated in Figure 8B. Using changes in the liquid gate voltage (V_{ref}), detection of RBD (Receptor Binding Domain of S1 subunit) of S protein could be achieved with a LOD of 0.2 pmol L^{-1} with a measuring time of only 2 min, according to Figure 8B.[24] The ACE2 enzyme could also be detected with S1 spike protein by monitoring the V_{ref} changes. This latter immunosensor is interesting for quantifications of infected cells, since ACE2, a human integral membrane protein, acts as receptor for SARS-CoV-2 binding and subsequent infection.[26] Considering the strategies involving different targets for detection published so far for SARS-CoV-2, we believe that monitoring real-time ACE2-spike S1 protein interactions is useful for evaluating *in vivo* infections.

In spite of being promising, electrochemical and electrical immunosensors frequently face selectivity as the main challenge for their validation. This is normally addressed with functionalization strategies, as mentioned above related to specific antibodies for COVID-19 early and sensitive diagnosis. However, the need of additional preparation steps for functionalization and biomolecules incubation frequently increases the biosensor fabrication time, making them more expensive than other immunosensing devices.

On the other hand, the adoption of functionalization strategies for selectivity improvement is also useful for minimizing interference effects. These methodologies are known for their significant efficiency, and consequently, they are adopted not only in biosensing technologies, but also in a wider range of detection through electrochemical methods. For instance, a gold microelectrode presented remarkable anti-interference activity toward As^{3+} detections by using amino-functionalized graphene oxide as modifying agent.[183] For SARS-CoV-2 diagnosis, interference from other CoVs with similar structures as well as other compounds from the sample matrix may be avoided by functionalizing the electrochemical device with recombinant antigens or their specific antibodies. This was the case of detection of SARS-CoV-2 S1 subunit antibodies using recombinant antigens on gold micropillar electrodes decorated with graphene oxide.[184] The LOD was 1 pmol L^{-1} with a detection time of 11.5 s with a smartphone-based interface,[184] but these devices may not be suitable for mass-testing because of the target analyte chosen by the authors: specific antibodies for S1 subunit proteins of SARS-CoV-2, which are not produced by a human infected organism. Instead, perhaps an electrochemical sensing platform for detecting IgG and IgM antibodies may be a more efficient strategy.

■ EMERGING STRATEGIES FOR DIAGNOSIS OF COVID-19

In this section, we wish to emphasize that the diagnosis of COVID-19 can be improved and extended if sensing strategies are explored, which can lead to low-cost methodologies for mass tests and/or increased accuracy. There are several such strategies, but for the sake of conciseness, we shall illustrate only three types: paper-based biosensing, plasmonic-enhanced biosensing, and use of pattern recognition methods (which may incorporate machine learning). All of these three strategies can be utilized not only for immunosensing and genosensing but also with alternative principles of detection in which specific biorecognition is not required.

Paper-Based Biosensors. Paper-based biodevices (PBBs) are simple, inexpensive, and robust alternatives to assays, as has been proven for diagnosis of infectious diseases.[185−187] These are miniaturized POC devices[188] made of porous cellulose capable of storing the reagents, and the reaction with the analyte being detected either visually by a color contrast through fluorescent/colorimetric dyes or via spectroscopic measurements.[189] In order to be sensitive and selective for a given disease, these devices have biorecognition elements immobilized, such as enzymes, antibodies, and aptamers.[190−193] The whole detection process is performed on a single strip of nonexpensive paper, without requiring additional instrumentation and multiple processing steps. This feature has been exploited in immunosensors[54,194,195] and in genosensors with nanomaterials such as reduced graphene oxide−gold nanocomposites[113] and bimetallic nanostructures.[48] For genosensors, sensitivity and accuracy for the diagnosis of malaria were higher than with RT-PCR.[196] PBBs are also useful for multiplex POC devices for sensitive, high quality diagnostics of biomarkers of several diseases.[186]

PBBs have been used to detect diseases within a label-free detection scheme in which a biorecognition element is not required. A PBB with oxidized paper was capable of detecting an abnormal level of human serum albumin (HSA) in urine samples via colorimetry, which is associated with a disease referred to as proteinuria/microalbuminuria.[197] Detection was made possible, in spite of the absence of a biorecognition element, owing to covalent bonding to the protein, as confirmed by micro-FTIR spectroscopy.

In spite of their advantages, PBBs have not been reported so far for SARS-CoV-2 diagnosis, probably because mass-testing in official centers of COVID-19 diagnosis is challenging. First, as already mentioned in the LFDs section, paper-based devices may be limited owing to their complexity.[158] Problems may arise from trapping of biomolecules, thus causing a decrease in binding effectiveness and requiring adequate preconditioning of the paper matrix.[157,158] Second, automated methods to fabricate paper-based devices need to be developed. This can be done with such techniques as inkjet printing, PDMS

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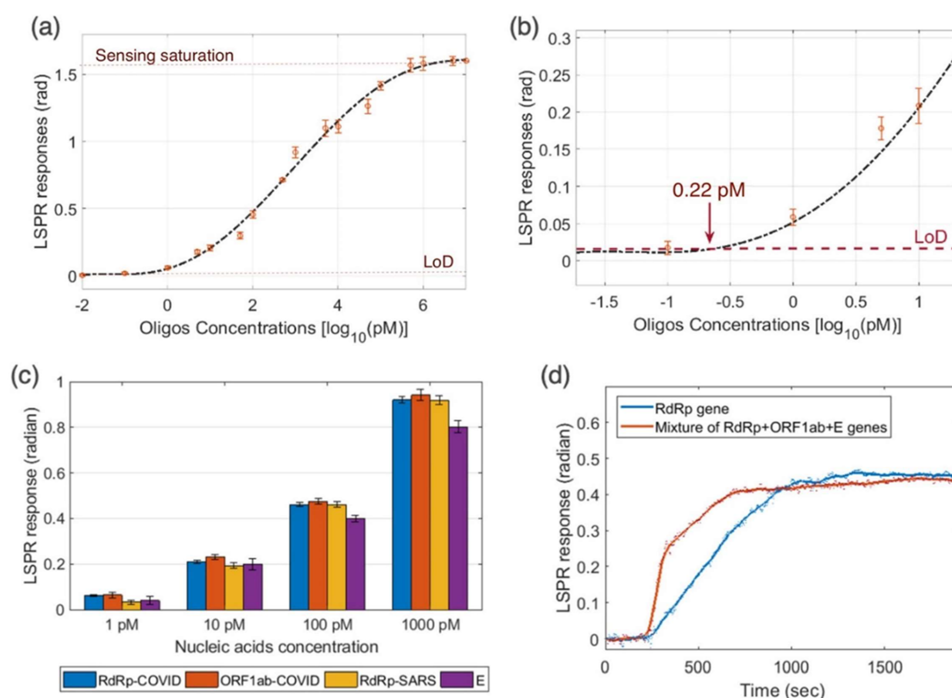

Figure 9. (a) LSPR response versus RdRp of SARS-CoV-2 concentration; (b) zoom of low-concentration region of LSPR biosensor responses for different RdRp oligos concentrations; (c) LSPR biosensor response for detection of other viruses, such as ORF1ab and E protein from SARS-CoV-2 and RdRp from SARS-CoV; (d) comparison of LSPR biosensor response in single-analyte samples and mixture of several sequences. Reprinted with permission from ref 215. Copyright 2020, American Chemical Society.

plotting, laser cutting, and photolithography. However, in addition to the expensive equipment required, preparation takes several steps and resolution is low.[198] Therefore, the scaling-up of PBBs may be less advantageous than expected, especially in the context of COVID-19 pandemics, which requires fast and effective strategies.

Plasmonic-Based Biosensors. Plasmonic effects have been exploited for biosensing with various principles of detection (for a focused review, see ref 199),[199] including surface plasmon resonance (SPR)[200−202] and localized surface plasmon resonance (LSPR).[203−205] LSPR, in particular, is promising owing to the enhanced electromagnetic field in the neighborhood of the nanostructures, which imparts higher sensitivity[206] as demonstrated in LFD assays where the colorimetric signal of gold nanoparticles was amplified upon laser excitation. Another advantage is the possibility of using laser-reader systems with the standard LFDs architecture and operation mode.[206] One should emphasize that LSPR can be useful for investigating interaction mechanisms during infection by SARS-CoV-2, including protein interactions with human receptors believed to be essential for the virus maintenance in human cells. Indeed, SPR can provide experimental evidence of phenomena related to docking studies.[207−210] Another advantage of LSPR biosensors is the cost of commercialization, as for example a home-built LSPR system based on white-light extinction would cost ∼$25,000 or less.[211] Compared with other more sophisticated LSPR equipment, this is a lower-cost possibility.

When plasmonic and thermal effects are combined, the sensitivity of the biosensing device for detecting nucleic acids can be increased considerably.[212] The "thermoplasmonic" effect arises from the nonradiative relaxation of absorbed light in nanomaterials, thus generating an excessive localized heat energy that can be harnessed as a local heating source for controlled thermal processes.[213] This provides a rapid alternative methodology for identifying DNA sequences and mutations.[214] A similar methodology was adopted in dual-function biosensors for SARS-CoV-2 detection[215] in synthetic viral oligonucleotide sequences identical to the one used in ref 53. The AuNIs (Au nanoislands) sensing chip functionalized with synthetic receptor oligonucleotide (RdRp SARS-CoV-2-C) was implemented into an LSPR detection system. Hybridization was ∼8 times faster with the thermoplasmonic effect when RdRp SARS-CoV-2 genes were injected into the sensing chamber. The evaluation of this dual-plasmonic device for viral nucleic acids detection based on LSPR responses is illustrated in Figure 9. It should be noted that nonspecific binding of mismatching sequences was prevented, which demonstrates the impact of localized heating on the hybridization kinetics. Various challenges must be addressed to apply LSPR biosensor to COVID-19, including the difficulties in preparing robust, reproducible substrates. Furthermore, the devices should ideally be reusable and easy to functionalize and clean after analysis.[211]

Artificial Intelligence Based Methods. The sensing strategies mentioned so far are all based on detecting specific targets requiring genetic sequence probes, proteins, and protein subunits. Because a considerable amount of data is generated, especially to account for the variability of the biological samples, statistical and computational methods can be utilized for the diagnosis, including machine learning, Internet of Things (IoT), deep learning, and other artificial intelligence (AI) approaches.[216−221] AI can also be employed during COVID-19 pandemics for the development of personalized medicine.[222] For example, Prasad and co-workers[223] developed a guide for reinforced learning

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algorithms to determine extubation time for each patient. The course of the treatment, evolution of symptoms, and day-to-day updates of the recuperation of patients can also be monitored through artificial intelligence algorithms.[224] Another prospective application of artificial intelligence is in the development of safe vaccines and pursuing therapeutic drugs.[225] Although artificial intelligence methodologies seem useful and efficient, several issues must be tackled for its widespread implementation. These include a speedy data collection, storage, and analysis, which requires specialized people and data centers. This may be difficult for some developing countries severely affected by SARS-CoV-2.

Perhaps the most important breakthrough will be reached when various types of data are integrated into a single system. This could be realized with computer-assisted diagnosis systems, as discussed in reviews dedicated to the use of Big Data concepts and computational methods in processing sensing data.[226–228] IoT approaches are also useful for online data collection from sensing devices and interpretation of these data, as well as orienting medical decisions based on Big Data analysis models.[221] The framework of these computer-assisted systems is entirely generic and can be applied to any type of task involving diagnosis, monitoring, or surveillance. More specifically for COVID-19, one may envisage a diagnosis strategy considering reports of symptoms, analysis of health parameters, and data from sensors which do not need to be specific for SARS-CoV-2 (i.e., sensors other than the immunosensors and genosensors discussed here). Hence, a somewhat accurate diagnosis could be achieved even if no specific tests are available. An added advantage would arise from feeding the results from a large number of people into a surveillance system to monitor the disease spread, which could be performed in a seamless manner in a computer system.

CONCLUSIONS

Herein we have described the main strategies employed so far in SARS-CoV-2 diagnosis, which are basically categorized into methods to detect genetic material of the virus and immunoassays. Special emphasis was given to identifying the strengths and limitations of these methodologies. In particular, we elaborated upon the limitations in connection with the difficulties in performing mass testing in many countries, from which we identified three major challenges: (i) employ genosensors in POC devices to replace the sophisticated methods used (RP-PCR, LAMP, CRISPR) in detecting genetic material, since this is critical to diagnose individuals with no symptoms; (ii) improve the accuracy of the diagnosis based on immunoassays, which is by no means straightforward because distinct types of antibodies may be needed to detect owing to the time dependence of their concentrations along the infection course; (iii) use pattern recognition methods that do not require biotech products and the test kits, which is essential in poorer places.

For all the challenges above, we envisage that new developments will be required in materials, especially nanomaterials. From our survey, we noted that the main emphasis has been placed on the definition of target biomolecules, but strategies to enhance performance using nanomaterials have been limited. For detection of genetic material, for instance, developing low-cost alternatives to the expensive methods should be prioritized, and this depends on novel uses of nanomaterials. One should aim at POC devices which can fulfill all the stringent requirements of low-cost, rapid tests and easy deployment in any setting. Also relevant to the chemistry and materials community is the possible extension of well-established electrochemical, electrical, and plasmonic effects to diagnosis of SARS-CoV-2. In this review, we have mentioned a few examples in which graphene and metallic nanoparticles were incorporated into the sensing devices, and we can foresee that much more can be done by exploiting the whole portfolio of biosensing strategies in which nanomaterials are used.

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Notes

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VOCABULARY

Biosensors: Biosensors are devices thar are able to transduce a biological event into a measurable analytical signal. Proteins, biological structures and microorganisms can be detected through biosensing strategies.

Antibody: An antibody is a protein produced by the immunological system for the defense of their host organism from external pathogens. They do it with high specificity, by binding to specific parts of the targets.

Point-of-care devices: These are devices that enable medical diagnosis at the time and place of patient care. They are suitable for quick and mass testing, without long sample preparation steps and reactions.

Genosensors: A genosensor is a specific type of biosensor dedicated to the detection of DNA (i.e., genetic material). The event that enables detection consists of hybridization of DNA specific probes that are complementary to the DNA (or RNA) target sequence to be detected.

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Electrochemical sensors: Devices that can give information on the composition of a system based on electron-transfer reactions into a sensing transducer (electrodes). The signal obtained can be used for quantification and other analytical purposes.
Analytical chemistry: A branch of chemistry dedicated to the determination of composition of matter and its quantification. In many cases, analytical chemistry deals with these issues through the development of robust instrumentation, implementation of statistics and innovative sensing strategies.

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