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Perspective

Paper-Based Biosensors for COVID-19: A Review of Innovative Tools for Controlling the Pandemic

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Cite This: ACS Omega 2021, 6, 29268–29290

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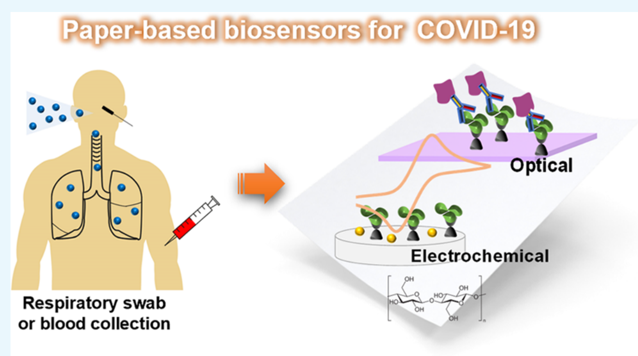
ABSTRACT: The appearance and quick spread of the new severe acute respiratory syndrome coronavirus disease, COVID-19, brought major societal challenges. Importantly, suitable medical diagnosis procedures and smooth clinical management of the disease are an emergent need, which must be anchored on novel diagnostic methods and devices. Novel molecular diagnostic tools relying on nucleic acid amplification testing have emerged globally and are the current gold standard in COVID-19 diagnosis. However, the need for widespread testing methodologies for fast, effective testing in multiple epidemiological scenarios remains a crucial step in the fight against the COVID-19 pandemic. Biosensors have previously shown the potential for cost-effective and accessible diagnostics, finding applications in settings where conventional, laboratorial techniques may not be readily employed. Paper- and cellulose-based biosensors can be particularly relevant in pandemic times, for the renewability, possibility of mass production with sustainable methodologies, and safe environmental disposal. In this review, paper-based devices and platforms targeting SARS-CoV-2 are showcased and discussed, as a means to achieve quick and low-cost PoC diagnosis, including detection methodologies for viral genomic material, viral antigen detection, and serological antibody testing. Devices targeting inflammatory markers relevant for COVID-19 are also discussed, as fast, reliable bedside diagnostic tools for patient treatment and follow-up.



INTRODUCTION

A novel human coronavirus, named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), emerged in December 2019, in Wuhan, Hubei Province, China. SARS-CoV-2, the etiological agent of COVID-19, spreads globally and rapidly, prompting a pandemic declaration by the World Health Organization (WHO) on March 11, 2020, resulting in upward of 120 million cases of infection and 2.5 million deaths up to date.[1] Coronaviruses belong to the subfamily *Coronavirinae* from the *Coronaviridae* family. *Coronavirinae* subfamily includes four genera of enveloped single-stranded positive sense RNA viruses, namely, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. SARS-CoV-2, as well as SARS-CoV and MERS-CoV (Middle East Respiratory Syndrome), belongs to *Betacoronavirus* genus.[2] SARS-CoV-2, as other coronaviruses, is an enveloped virus, with a viral genome enclosed in an helicoidal capsid. Spike (S), membrane (M), and envelope (E) proteins are the principal viral envelope proteins, while nucleocapsid (N) protein, along with RNA molecule, composes the viral nucleocapsid[3] (Figure 1A).

Binding and entry of SARS-CoV-2 in host cells depends on the densely glycosylated S protein, which contains two major subunits; the receptor binding domain (RBD) (S1), responsible for virus attachment to the host cell receptor, such as angiotensin-converting enzyme 2 (ACE2), although other interactors are probable, and a second domain (S2) that mediates the viral fusion with the host cell membrane.[4] Therefore, key steps of SARS-CoV-2 infection include the identification of target cells, the maturation of protein S, and ultimately, virus cell entry. In more detail, attachment to host cells takes place *via* binding of the viral S protein to ACE2, the viral receptor on host cells. After this first step, an initial (priming) cleavage event occurs between S1 and S2 domains by host cell proteases, namely, by furin, PC1 (prohormone

Received: July 27, 2021
Accepted: October 18, 2021
Published: October 29, 2021

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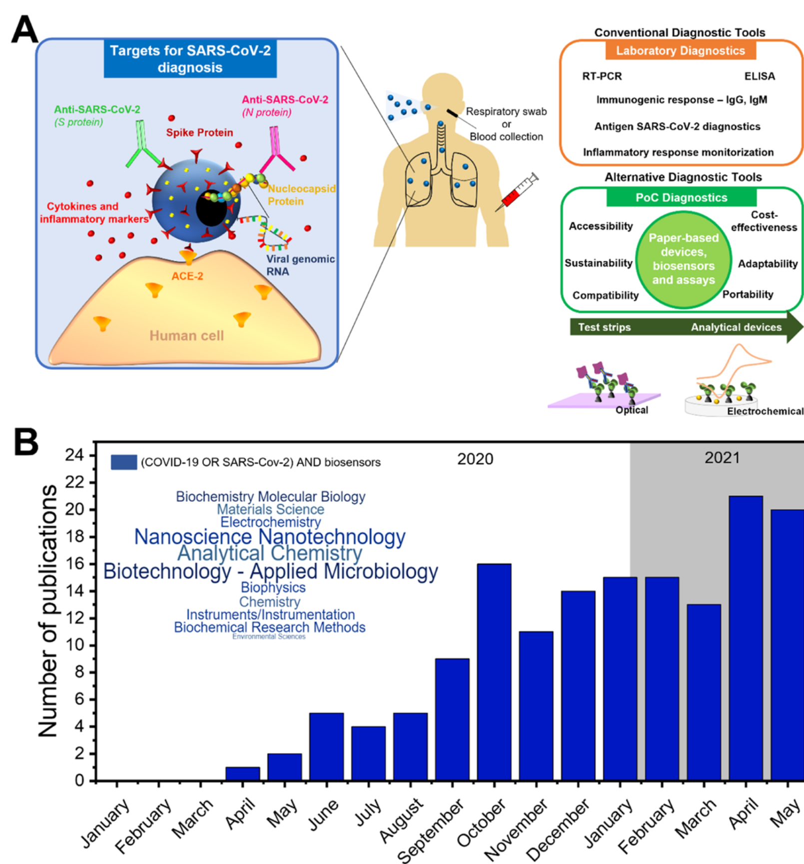

Figure 1. (A)Targets for SARS-CoV-2 diagnosis include viral genomic material and specific viral antigens, namely, the spike and nucleocapsid proteins, as well as organism response markers to the presence of the virus, through antibody production and inflammatory response. Respiratory samples or serological samples are used for identification of these markers, with complementary laboratory techniques or alternative diagnostic tools and point of care devices, where paper-based alternatives present themselves as attractive, affordable and more accessible tools. (B) Statistics on number of publications related to biosensing developed for SARS-CoV-2 and COVID-19, retrieved from PubMed database (query: (COVID-19 OR SARS-CoV-2) AND biosensors). Data were retrieved from the date June 15th 2021.

convertase 1), trypsin-like proteases, cathepsins, and the serine proteases TMPRSS2 and TMPRSS4 (transmembrane serine proteases 2 and 4, respectively). The proteolysis produces a mature S2 fusion protein, enabling the fusion of viral and cellular membranes and the consequent entry of viral RNA into the host cell.[3,4] Once the nucleocapsid is deposited into the cytoplasm of the host cell, the RNA genome is replicated and translated into structural and accessory proteins. Vesicles containing the newly formed viral particles are then transported to and fused with the plasma membrane, releasing them to infect other host cells, using the same infectious strategy.[4] Of relevance, SARS-CoV-2 RBD has been demonstrated to have 10- to 20-fold increased binding affinity to ACE2 compared to SARS-CoV RBD. It is currently well known that SARS-CoV-2 is mostly transmissible through large respiratory droplets, directly infecting cells of the upper and lower respiratory tract, especially nasal ciliated and alveolar epithelial cells. However, in addition to the lungs, ACE2 is also expressed in several human tissues and organs, such as kidneys, heart, the small intestine, thyroid, adipose tissue, and testis, suggesting that the virus may directly infect cells of other organ systems when viremia is present.[2−4]

For medical diagnosis of this pandemic virus, detection approaches targeting its genetic material have become the gold standard for case identification and diagnosis, with nucleic acid amplification testing (NAAT), namely, real-time RT-PCR (reverse transcriptase-polymerase chain reaction), due to the possibility for highly sensitive and specific assay performance that allows for qualitative, early infection identification in a high-throughput, laboratorial setting.[5,6] Although current gold-standard methodologies for SARS-CoV-2 viral RNA identification have been readily employed worldwide and have been instrumental for reliable infection identification,[7,8] they pose impediments toward wide testing of people as a key action to control the spread of the virus.[9] These methods rely on specific reagents that may be readily available in some countries, but may lack in other areas of the world, creating a gap between the need for testing and the availability of currently, very highly needed materials,[10] as well as on specialized personnel and equipment able to carry out these tests.[11] They are also time consuming and performed in a centralized fashion, usually with different collection and test performance locations, increasing the time until the final test result, limiting contact tracing and treatment of patients that in some cases will only perform tests after symptom onset.[12,13] Other analytical limitations, such as the possibility for false positives and negatives that have been observed, or differences between test kits' analytical performance have been a concern regarding these nucleic acid amplification diagnosis assays.[14]

Thus, there is a stringent need for fast, accurate, and accessible tools for controlling this rapidly evolving pandemic or others that may come, where rapid testing in large scale is of

https://doi.org/10.1021/acsomega.1c04012
ACS Omega 2021, 6, 29268−29290

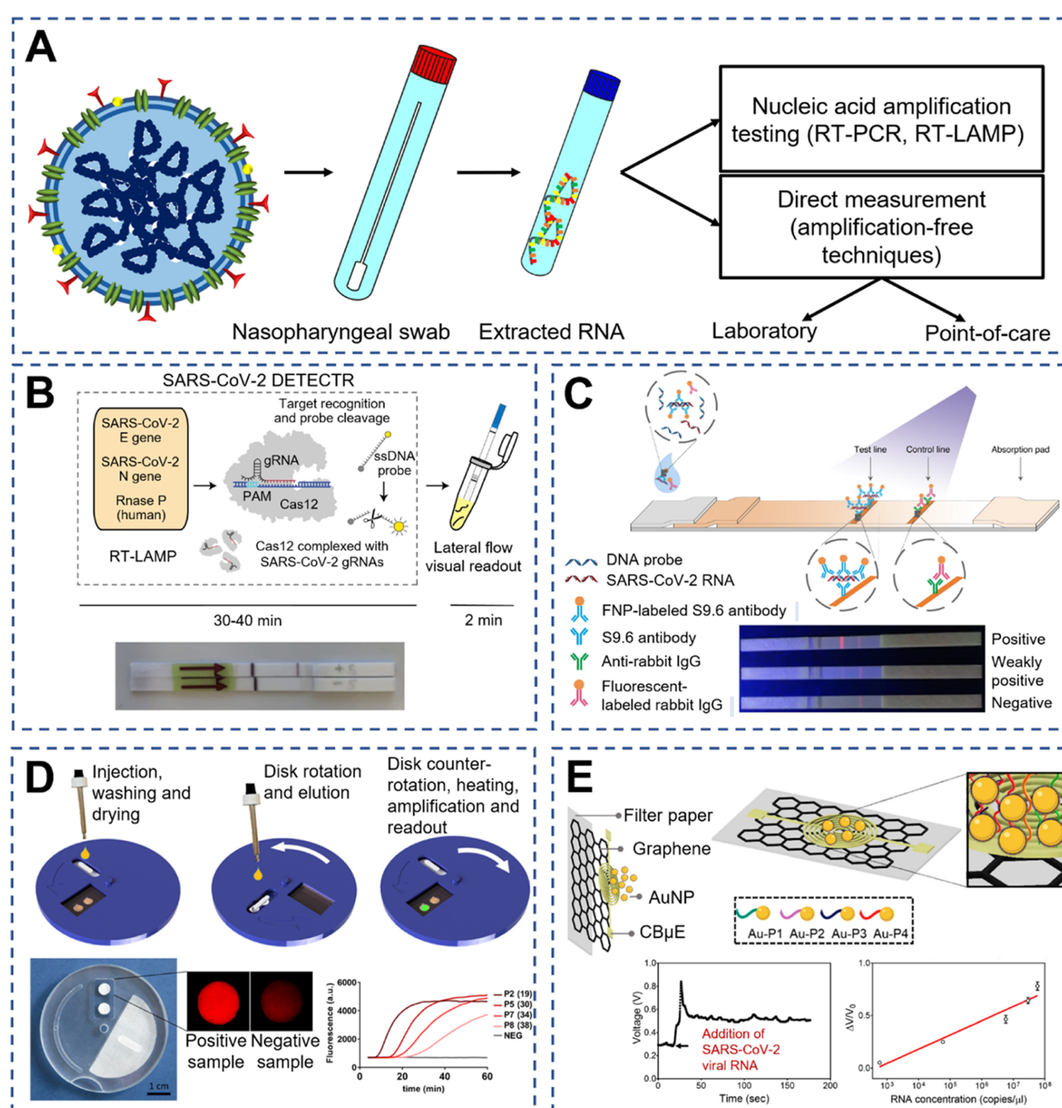

Figure 2. Viral genetic material identification and diagnosis methods in paper-based formats. (A) Workflow of sample collection and treatment for subsequent detection methodologies for RNA-based diagnosis. (B) CRISPR-based SARS-CoV-2 RNA identification with paper-based, LFA signal readout. Adapted with permission from ref 69. Copyright 2020 Springer Nature. (C) Fluorescence LFA format device for amplification-free RNA detection. Adapted with permission from ref 73. Copyright 2020 Springer Nature. (D) RT-LAMP RNA detection in a microfluidics-based, multifunctional device, carrying multiple tasks of the molecular diagnosis assay. Adapted from ref 75, copyright Garneret *et al.* (E) Electrochemical, paper-based platform for amplification-free, viral genomic identification and diagnosis, using ssDNA probes and graphene/Au electrodes modified with conjugated AuNPS. Adapted with permission from ref 88. Copyright 2020 American Chemical Society.

great importance to restrain the disease spread.[15] In this context, point-of-care (PoC) testing devices and biosensors can give a significant contribution to the set of diagnostic and virus identification tools already in action,[16] represented by the increased number of publications related to biosensors targeting SARS-CoV-2 (Figure 1B). These devices are more simply operated and interpreted, with improved cost-effectiveness and more easily employed in areas with lower accessibility to centralized healthcare, either far from bigger urban centers or underdeveloped areas of the world, where well-equipped laboratories are less common.[17] Similarly, to the development of therapeutic approaches and vaccine development for SARS-CoV-2, there is a challenge of rapidly investigating and developing new highly accessible biosensing tools that bring multidisciplinary contributions in PoC use.

From the several approaches in PoC, biosensors employing cellulose and its derivatives as a substrate are particularly interesting as they benefit from the prompt availability of cellulose and the possibility of an environmentally safe discard, thereby contributing for the global massive testing requirements under pandemic conditions. There are many approaches that have been developed in time to achieve a successful biosensor on cellulose-based substrates,[18−24] including cellulose of bacterial origin,[25] much before COVID-19, and they consist of immobilizing specific reagents on these substrates. Generally, this includes reagents that react with a given compound or group of compounds,[26] metal nanoparticles (NPs) with catalytic or plasmonic features[27,28] or selective biorecognition elements that capture a wide range of target analytes,[29−31] including disease diagnosis.[32−35] These events may be translated by monitoring alterations in optical features,[36] which may involve changes in maximum wavelength reflection/absorption[37] or the enhanced Raman-scattering properties.[38,39] Translation by electrochemical properties has

also been achieved[40−42] by having conductive inks casted on a cellulose substrate[43] or by hand-painting these inks[44,45] or using a printer for the same purpose.[46] Although the most well-known cellulose-based PoC approaches are lateral-flow methods,[47,48] with a well-known mode of operation that may be translated by naked-eye, optical, or electrochemical detectors. Other operating forms of paper-based sensors are also common among the literature and include microfluidics on paper[49,50] or simple dipping/casting methods, as the common pH paper that exist in the laboratories[26] or the glucose meter made on a paper substrate.[29,31] Only 1 year after the pandemic, many biosensing approaches have been developed in the context of COVID-19,[51−54] and these also include cellulose-based approaches that deserve being highlighted and suitably reviewed.

Although being a routinely used material in the society, there are many forms of cellulose paper and its chemically-derived materials used in biosensors, also including nanostructured organizations of cellulose,[55] or important derivatives, as nitrocellulose, widely employed in the design of lateral flow methods.[56] Such a wide variety of compounds, allied to a wide range of processing methods, allow for the fine tuning between porosity, fiber size, mechanical strength, thickness, capillarity and associated hydrophobic or hydrophilic properties, and the intended sensing application. Overall, cellulose presents attractive features powering its suitability for applications in analytical devices, namely, its biocompatibility with various bioassay reagents, capability for chemical modifications and functionalization, and high thermal and mechanical strength,[57] withstanding many different manufacture processes required for analytical device fabrication.[58,59] Consequently, the paper can play different active roles in these analytical devices, going from a structural platform for immobilization and fabrication of active structures (*e.g.,* electrode fabrication),[60] container for reagents and samples or actively transporting them through capillary forces,[58] allowing for sampling,[61,62] filtration,[63] chromatographic separation,[64] and reagent/sample mixing.

Much of these aspects related to the paper are potentiated by the different architectures that can be used for analytical device production. The most used cellulose-based device is the lateral flow test strip that finds many applications for immunoassays. These devices are attractive due to their low cost and user friendliness, generally with a colorimetric signal output, easily read by visual examination or portable readers, or even smartphones, for both qualitative and semi-quantitative information being retrieved from a specific test. To further enhance the functionalities of devices produced from the paper, microfluidic paper-based analytical devices (μPADs) have been introduced. From the extensive array of different paper-based test formats, paper presents itself as one of the most suitable materials to tackle the need for accessible, lower-cost biosensing devices in the context of the SARS-CoV-2 pandemic and its requirements for diagnosis and disease management, in addition to control of virus spread. In a moment where vaccine development has shown rapid progresses, there is also the need for fast serological screening of immunological response in both infected and inoculated people, which is able to determine the acquired immunity that may be the answer for definite control of the virus. Furthermore, paper-based platforms may find other implementations in epidemiological early warning, aiding in early identification of virus outbreaks in different settings, while meeting some of the goals for sustainable development proposed by the United Nations.[65] In this review, paper-based devices and biosensors designed for viral detection, immune response detection, and epidemiological applications in the context of SARS-CoV-2 pandemic are addressed and discussed, in terms of their suitability and potential to complement established pathogen detection methods and aid in the collective effort against this pandemic.

2. PAPER-BASED DIAGNOSTICS FOR VIRAL PATHOGEN DETECTION

2.1. Paper-Based Biosensors for Viral RNA Sensing. SARS-CoV-2 identification and detection has been routinely performed by sensing viral genetic materials, antigen structures, and/or the whole virus. The later approach related to the detection of specific genome sequences, responsible for encoding specific viral structures, in nasopharyngeal specimens (Figure 2A), such as the envelope protein (E gene), RNA-dependent RNA polymerase (RdRp gene), or nucleocapsid protein (N gene), has been performed by reverse transcription of viral RNA.[66] Depending on the target genomic sequence used, the analytical performance of the test varies, showing variable specificity or sensitivity toward SARS-CoV-2. This variability is a critical feature, considering that viral loads associated with this virus show significant variation.[67] Overall, these requirements need to be considered when developing new PoC paper-based sensing devices to be used in different detection tasks, opening the possibility for integration of standard, ultrasensitive biotechnological techniques for molecular diagnosis that need to be able to detect a minimal numbers of copies.[68]

In this setting, lateral flow assays (LFAs) have emerged as an effective approach to produce portable, low-cost diagnostics, without the need for qualified individuals and specific laboratorial equipment. This follows the previous extensive development of LFAs in a wide range of applications[48] In the context of SARS-CoV-2, LFA test strips composed of nitrocellulose have been introduced to viral RNA detection, following the early evidence of this technology in the development of highly specific biochemical sensing approaches. More specifically, novel biosensing approaches using genetic material amplification and gene editing techniques have appeared in response to the diagnostic needs in the pandemic situation, gaining high preponderance both in research and clinical settings. Broughton *et al.*[69] presented an LFA able to detect E and G genes using reverse transcription loop-mediated isothermal amplification (RT-LAMP) pre-amplification, paired with clustered regularly interspaced short palindromic repeats (CRISPR-cas12) genome editing technology (Figure 2B). Using nasopharyngeal swabs, followed by viral RNA extraction, these techniques were used to amplify RNA strands that can be detected by fluorescence measurements or visually in a test strip, with the total process reducing test time to be around 45 min, with a limit of detection (LOD) of 10 copies/μL. Other similar approaches were presented by Curti,[70] Joung,[71] and Patchsung *et al.*,[72] where the application of CRISPR-mediated detection paired with different amplification approaches was used to detect synthetic SARS-CoV-2 RNA materials, N gene, open reading frame 1ab (*Orf1ab*), and other relevant genomic sequences in saliva and nasopharyngeal samples, using an LFA format. These techniques allow for highly specific and sensitive sensing of SARS-CoV-2, in a low-cost, portable fashion, that

https://doi.org/10.1021/acsomega.1c04012
ACS Omega 2021, 6, 29268−29290

Table 1. Paper-Based Assays for Viral Genomic Material Sensing Schemes and Attained Analytical Performance

transduction	scheme	format	target RNA	LOD	reference
fluorescence	LAMP pre-amplification + CRISPR cas12a + biotin-labeled ssDNA reporter	LFA	N and E genes	10 copies/μL	69
	RT-RPA + CRISPR cas12a + biotin-labeled ssDNA reporter	LFA	RdRp, ORF1b, ORF1ab	10 copies/μL	70
	DNA probes + fluorescence-NP labeled monoclonal antibodies	LFA	N, E, Orf1ab genes	1000 TU/mL	73
colorimetric/fluorescence	LAMP + CRISPR cas12 + biotin-labeled ssDNA reporter	LFA	N gene	100 copies/reaction	71
	RT-RPA + CRISPR cas13 + biotin−fluorescein RNA reporter	LFA	S, N, Orf1ab genes	42 copies/reaction	72
	LAMP + intercalating dyes/fluorescence probes	Microfluidic	RdRP gene	1 copies/μL	75
colorimetric	RT-LAMP + streptadivin coated particles + capture antibodies	LFA	N and ORF1ab genes	2 copies/μL	89
	RT-RAA + biotin + FITC	LFA	N gene	1 copies/μL	90
	CRISPR/Cas9 + AuNPs-DNA probes	LFA	E and ORF1ab genes	100 copies/reaction (25 μL)	91
	AuNP-detector probes + capture probes	LFA	ORF1ab	0.5 nM	92
electrochemical (potential variation)	ssDNA probes conjugated with AuNPs for electrode modification (graphene + Au μelectrodes)	Electrical sensor chip	N gene	6.9 copies/μL	88

could be deployed worldwide to complement conventional RT-PCR detection, since the presented LODs are well below the viral genetic content in samples from infected patients. Other approaches for viral RNA identification can also be used in LFAs, without the need for amplification steps. Wang *et al.*[73] used DNA probes for the specific and sensitive identification of N and E genes and *Orf1ab*, paired with fluorescent NPs labeled with antibodies for signal transduction in test lines (Figure 2C). Without any amplification step, a LOD of 1000 TU/mL was achieved by this assay, still comparable to the ones presented by RT-PCR technology.

Besides the conventional LFA format, other paper-based approaches were proposed for the performance of these biochemical assays. In a perspective article, Yang *et al.*[74] presented the concept of LAMP in paper for SARS-CoV-2 viral RNA sensing, discussing the necessary requirements to achieve a PoC paper-based colorimetric diagnostic device. Another work based on this approach was presented by Garneret *et al.*[75] Exploiting the potential of paper microfluidics to develop multifunctional devices able to perform multiple reaction steps in a single platform, a LAMP-based assay was adapted to target RdRP gene in nasopharyngeal swab samples from infected patients (Figure 2D). The COVIDISC device was able to incorporate all the reaction steps of the LAMP assay (extraction, elution, reverse-transcription, amplification, and transduction), leading to measurable fluorescence signals. These signals could also be detected by the naked eye.

This approach was also tested against eight other major respiratory viruses, to portray the high specificity of these assays, showing no cross-reactivity. Such devices show great potential for application in PoC settings, as cost-effective, easy-to-use alternatives with comparable analytical performance to standard methods. Furthermore, they show great versatility for application toward the detection of many different pathogenic viruses (*e.g.,* dengue, ebola, zika),[76−79] a feature that is particularly relevant because new virus mutations and strains are being identified, and may need more specific detection methods. However, these approaches suffer from some of the disadvantages of conventional techniques because they rely on similar biological reagents to conventional RT-PCR (*e.g.* DNA probes, primers, fluorescent reporters) that may not be readily available worldwide. To circumvent such limitations, test kits containing all the necessary reagents and materials need to be manufactured, so that these assays can be quickly employed, while also needing rapid approval, sometimes with emergency use authorizations. After meeting all these requirements, some of the presented approaches have reached practical application for PoC diagnostics, having rapidly reached the market.[80]

Another aspect of concern in SARS-CoV-2 diagnosis is the novel and emerging viral strains that brought different concerns regarding the transmissibility and subsequent consequences of SARS-CoV-2 infection.[81] Thus, it is important to not only to detect and diagnose infections but also identify these strains, to mitigate their greater infectivity and severity, where novel sequencing techniques may play a significant role.[82] Taking this into account, some works have been put forward to apply such approaches for rapid and accurate strain identification and associated mutations, using paper-based methodologies. Some of these mutations occur in the S gene, and are present across multiple variant lineages, prompting Kumar and co-worker to develop a CRISPR-based diagnostic paper test strip for the identification of N501Y mutation, using *Francisella novicida* Cas9 enzyme.[83] This assay is not only capable of SARS-CoV-2 infection detection but also for the detection of this mutation, with the potential for adaptability toward other mutations of interest. Using infected patient samples, sensitivity and specificity of 87 and 97% were obtained. Similarly, other mutations and single nucleotides associated with influence in specific variants have been targeted by Osborn *et al.*, as a proof-of-concept for CRISPR/Cas9 applied to LFA, showing that signals could be generated to detect DNA targets at single nucleotide level.[84] Such approaches could show great utility in providing rapid information on the prevalence of different viral strains and could help complement more complex, laboratorial sequencing techniques that are not fully accessible worldwide.

Paper-based electrochemical biosensors are also useful alternatives for the detection of viral genetic material. Approaches based on electrochemical transduction have been employed for the detection of many viruses, using different printing techniques for electrode fabrication and subsequent modification with nucleic acid probes or nanostructures with affinity toward the target viral genetic material. These approaches have been presented by Teengam *et al.* for

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ACS Omega 2021, 6, 29268−29290

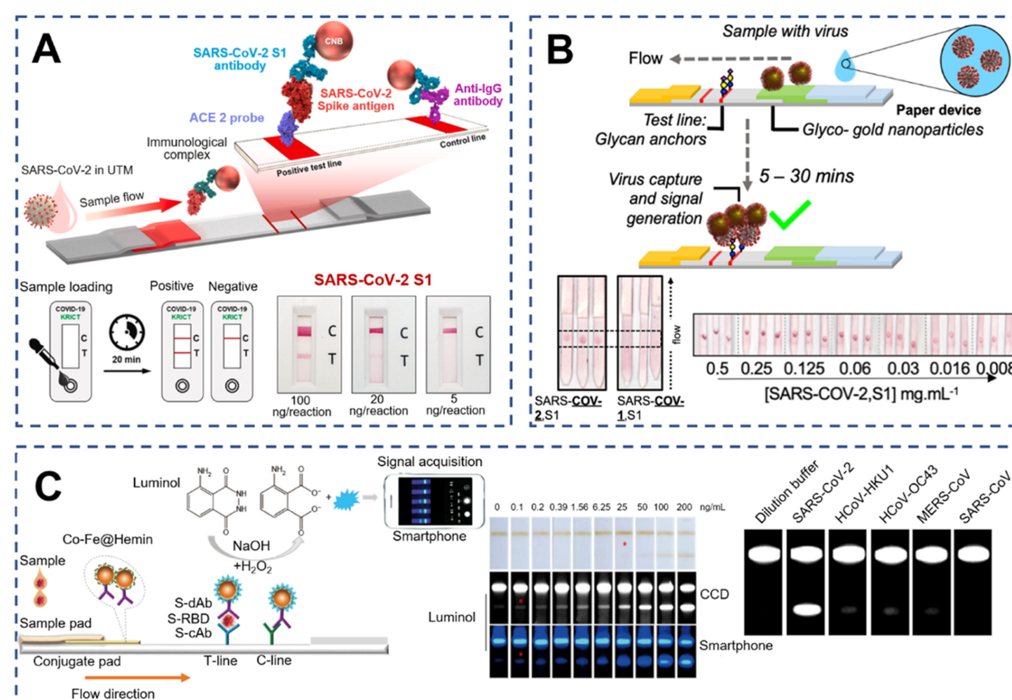

Figure 3. SARS-CoV-2 spike antigen detection employing paper-based platforms. (A) Spike antigen detection in LFA format, employing ACE2 as capture molecule for flowing immunocomplexes. Adapted with permission from ref 96. Copyright 2021 Elsevier. (B) Spike antigen detection in LFA format, using the natural binding path of this antigen to sialic acid, removing the need for antibodies and immunoassay-based detection of this antigen. Adapted with permission from ref 97. Copyright 2020 American Chemical Society. (C) LFA device for spike antigen detection, employing nanozymes and chemiluminescence signaling, for portable reading using smartphone readout. Adapted with permission from ref 98. Copyright 2021 Elsevier.

human papillomavirus,[85] by Narang *et al.* for herpes virus,[86] and by Singhal *et al.* for chikungunya[87] viral genetic detection, using current response for correlation with nucleic acid copy levels. For SARS-CoV-2, most of these electrochemical transduction methods have focused on amplification-free approaches, using standard recognition elements such as oligonucleotides and aptamers capable of binding to target viral RNA, showing the applicability of standard detection methodologies toward the development of biosensors toward SARS-CoV-2 detection. Alafeef *et al.*[88] have introduced a paper-based electrochemical sensor chip capable of targeting the N-gene with high specificity and sensitivity, with a greatly reduced assay time when compared with conventional biochemical sensing techniques, of less than 5 min (Figure 2E). This sensor is based on the modification of a chromatography paper substrate with graphene nanoplatelets, followed by electron beam deposition of gold microelectrodes. These electrodes were then modified with gold NPs (AuNPs) conjugated with highly specific antisense oligonucleotides with affinity towards the N gene. By measuring the difference in voltage upon the introduction of nasopharyngeal swab samples, the sensor was able to distinguish between positive COVID-19 patient samples and healthy asymptomatic samples with outstanding accuracy (near 100%), reporting a LOD of 6.9 copies/μL. Furthermore, the presented approach showed excellent specificity, reporting no significant electrical response when in the presence of MERS-CoV and SARS-CoV. These paper-based electrochemical approaches show great promise for low-cost, user-friendly, quantitative detection because they do not require pre-amplification steps, thereby opening the possibility of direct testing of samples at the PoC, using integrated and portable mobile readers for rapid diagnosis. Moreover, this principle may be extended to the detection of other viruses or viral strains. A comparison between different sensing schemes for viral genomic diagnosis using paper-based format platforms is shown in Table 1, comparing the transduction methods and analytical performance of the proposed sensing methods.

2.2. Paper-Based Biosensors for Viral Antigen Sensing.

An alternative approach for SARS-CoV-2 identification is the detection of viral antigens specific to this virus, namely, the N phosphoprotein and S glycoprotein. These viral structures are useful in early diagnosis and detection of circulating virus in the organism. Biosensors targeting the S protein in relevant body fluids open the possibility for a direct detection of whole virus particles[93] and in the case of protein N, the identification of infection, before symptom onset and immune response by the organism.[94,95] As for the detection of genetic materials, one of the first approaches that was readily employed for the detection of these analytes was LFA test strips. Multiple works have been presented for the detection of these protein structures, mostly employing target specific antibodies or other receptors, immobilized onto the nitrocellulose membrane. Conjugated, reporter particles, which are able to bind to the target analyte and carry it into the test lines were used to produce colorimetric outputs, signaling the presence of the antigen. In this antigen detection approach toward SARS-CoV-2 diagnosis, some standard detection techniques were successfully applied and translated to the market, including the conjugation of reporter particles with anti-SARS-CoV-2 antigen antibodies and the immobilization of these antibodies into the nitrocellulose membranes. Simultaneously, new detection schemes were successfully developed, based on specific biochemical behaviors of these antigens or by

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ACS Omega 2021, 6, 29268−29290

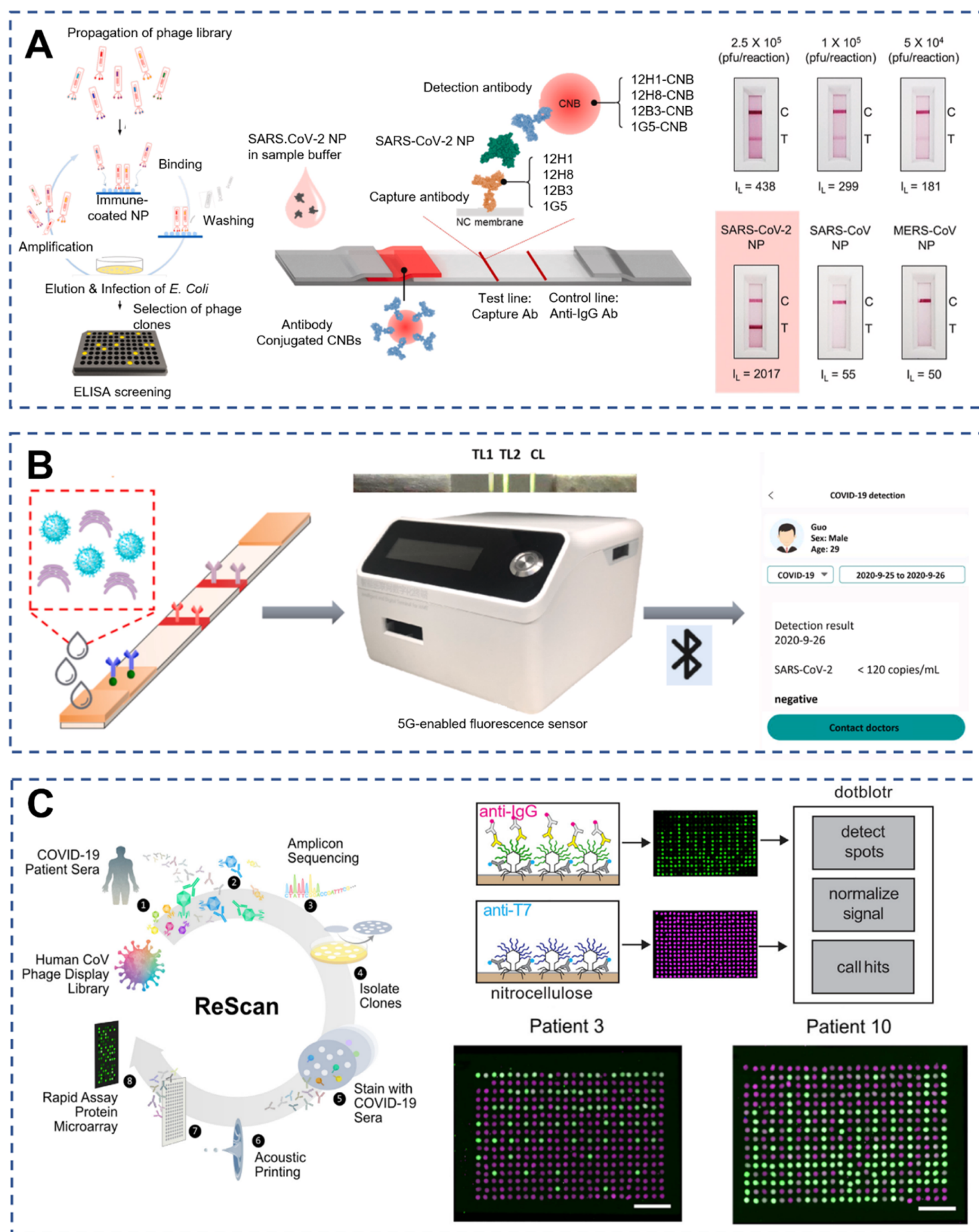

Figure 4. SARS-CoV-2 nucleocapsid antigen detection or simultaneous spike and nucleocapsid antigen detection employing paper-based platforms. (A) Nucleocapsid antigen detection using LFA system built using fusion antibodies produced from cloned phages. Adapted with permission from ref 100. Copyright 2021 Elsevier (B) Spike and nucleocapsid simultaneous antigen detection using LFA system with up-converting nanoparticles and a 5G-enabled fluorescence sensor for IoT applications. Adapted with permission from ref 101. Copyright 2021 Elsevier. (C) High-throughput, proteomic detection of SARS-CoV-2 S and N antigen and their subunits, by printing of cloned phages expressed antibodies targeting the antigens, in nitrocellulose substrate. Adapted with permission from ref 102. Copyright 2020 Elsevier.

employing alternative transduction and reading schemes, both for S and N protein detection. Some of these new biosensing schemes, specific to SARS-CoV-2 S protein detection include the work by Lee *et al.*,[96] where the ACE2 receptor was paired with red cellulose nanobeads conjugated with monoclonal antibodies to bind to the S antigen. An ACE2 receptor was

immobilized on the nitrocellulose membrane, acting as a capture probe for flowing immunological complexes comprised of the spike antigen (S1 unit) and its specific monoclonal SARS CoV-2 S1 antibody (Figure 3A). This LFA presented a LOD of 1.86×10^5 copies/mL in clinical COVID-19 positive specimens, with no cross-reactivity toward other spike protein S1 units from other coronaviruses. Another approach for LFA-based detection of antigens was proposed by Baker *et al.*,[97] where the glycan-binding properties of the spike protein were used as a detection method (Figure 3B). Using glycopolymer-functionalized AuNPs and glycan anchors at the test line, more specifically sialic acid derivatives, capture of the S1 subunit of the spike protein was achieved using virus mimicking particles bound to the antigen. This work showed that by applying a natural binding pathway of the virus, such as sialic acids, found in the human respiratory tract and crucial for engagement of the virus with host cells, antibody-free approaches for rapid testing can be developed. Another approach applied in LFA testing was presented by Liu *et al.*,[98] based on the use of nanozymes with catalytic activity able to amplify the immune reaction signal (Figure 3C). Using Co−Fe@hemin nanocomposites, peroxidase activity was applied for magnifying signals provided by standard immunoassay, with capture antibodies conjugated with the nanocomposites and immobilized at test lines in the nitrocellulose membrane. By means of chemiluminescence, signals were detected by smartphone imaging with improved sensitivity, when compared to conventional colloidal gold or fluorescence-based detection, with a decreased assay time of 16 min, showing good prospects for application in PoC.

For the detection of N protein antigen, Grant *et al.* presented a half-strip LFA developed using commercially available materials, with colored latex beads as signal transducers.[99] Using the target selective polyclonal antibodies for conjugation with latex beads and binding of immunocomplexes at the test line, N antigens from two different suppliers were detected with low LODs, in the ng/mL range. Alternatively, Kim *et al.* presented the application of specific single-chain variable fragment-crystallizable (scFv-Fc) fusion antibodies for LFA testing,[100] as shown in Figure 4A. These scFv-Fc antibodies were produced using a phage display technology and were then selected for N protein antigen detection. These scFv-Fc antibodies were used as capture antibodies in the nitrocellulose membrane, while simultaneously conjugated with cellulose nanobeads (CNBs) for detecting the immunocomplex formation. Using this approach, sensitive and highly specific antibodies were synthesized, able to detect N antigen with a LOD of 2 ng/reaction (20 ng/mL). Furthermore, this system was also able to detect cultured virus in a lysis buffer, with a LOD of 2.5×10^4 pfu/reaction. To showcase the applicability of these devices and assays for PoC applications, integrated with internet of things (IoT) functionalities, Guo *et al.* implemented innovative immunoassay test strips with 5G-enabled fluorescence detection.[101] They were able to rapidly disseminate test results into smartphone applications or centralized healthcare facilities, following the operation principle represented in Figure 4B. Using up-conversion NPs combined with mesoporous silica ($UCNPs@mSiO_2$) conjugated with monoclonal antibodies, a single test strip was developed to detect both S and N protein antigens, simultaneously. With the 5G-enabled fluorescence sensor, the signals generated by the immunocomplexes resulted in low LODs, of 1.6 ng/mL for S antigen and 2.2 ng/mL for N antigen.

Other formats have been presented for specific antigen profiling in relevant human samples. Zamecnik *et al.* presented a high-throughput diagnostic method for proteome profiling, able to identify both S and N protein antigens in serum samples.[102] The ReScan method was proposed by printing of cloned phage expressing antibodies targeting the specific antigens, into a paper-based, nitrocellulose microarray (Figure 4C). In this microarray, dot blot type assays were able to differentiate between different antigens, namely, various S and N protein fragments and subunits, as well as other relevant protein structures. The authors claimed this as a useful tool, not only for diagnostic but also for profiling valid biomarkers in other infectious and autoimmune diseases, which is also important in the current setting, where new SARS-CoV-2 viral strains are being identified and spreading around the world.

Taking this into account, some developments have been presented for the detection and differentiation between different coronaviruses and SARS-CoV-2 variants, based on these paper-based antigen profiling platforms. Because these variants differ mostly from their S protein, Hristov and co-workers developed paper-based immunoassays in the LFA format, with the purpose of differentiating S proteins from distinct coronaviruses (SARS-CoV-1, SARS-CoV-2, and CoV-HKU1), which are also able to distinguish fraction of specific spike proteins.[103] Similarly, Tan and co-workers have used the potentialities of LFAs for the performance of immunoassay designed to characterize and select SARS-CoV-2 RBD-specific antibodies, thus being able to identify several viral strains, namely, the B.1.1.7 or Alpha, B.1.351 or Beta and P.1 or Gamma variants.[104] Although the main goal of the work was to determine the binding and neutralizing capabilities of the selected antibodies, one could envision the possibility of fabricating LFA diagnostic devices or others with the capability of detecting specific variant RBDs and antigen variations for SARS-CoV-2 strain differentiation upon patient diagnosis.

Amongst optical transduction mechanisms, surface enhanced Raman spectroscopy (SERS) combined with paper-based analytical devices can also represent a valuable approach for COVID-19 diagnosis. This technique allows for label-free detection by measuring the Raman intrinsic signal of analytes, decreasing the overall cost and the complexity of assays. Moreover, more complex detection schemes can also be followed by SERS, such as immunoassays, using the Raman reporters or by the observation of changes in the obtained spectra. SERS detection schemes can provide sufficient enhancement for single-molecule detection, combined with a simple assay protocol and fast response[105,106] and several paper-based SERS analytical platforms have been reported for multiple analytes, with outstanding performances in terms of specificity and sensitivity, reproducibility and stability.[28,38,39,107−110]

The ability of SERS as a tool for virus detection is well documented in the literature. From label-free approaches for simultaneous virus capture and detection[111,112] to immunoassay methods,[113,114] SERS has proven to be a promising technique for routine virus screening. Paper-based SERS devices for virus monitoring have also been reported, showing the potential of this approach in COVID-19 diagnosis.[115] Preliminary works on SARS-CoV-2 SERS-based screening have been published, attaining LODs in the order of magnitude of 10^{-9} mol/L.[116,117] In this context, Jadhav *et al.* proposed a

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ACS Omega 2021, 6, 29268−29290

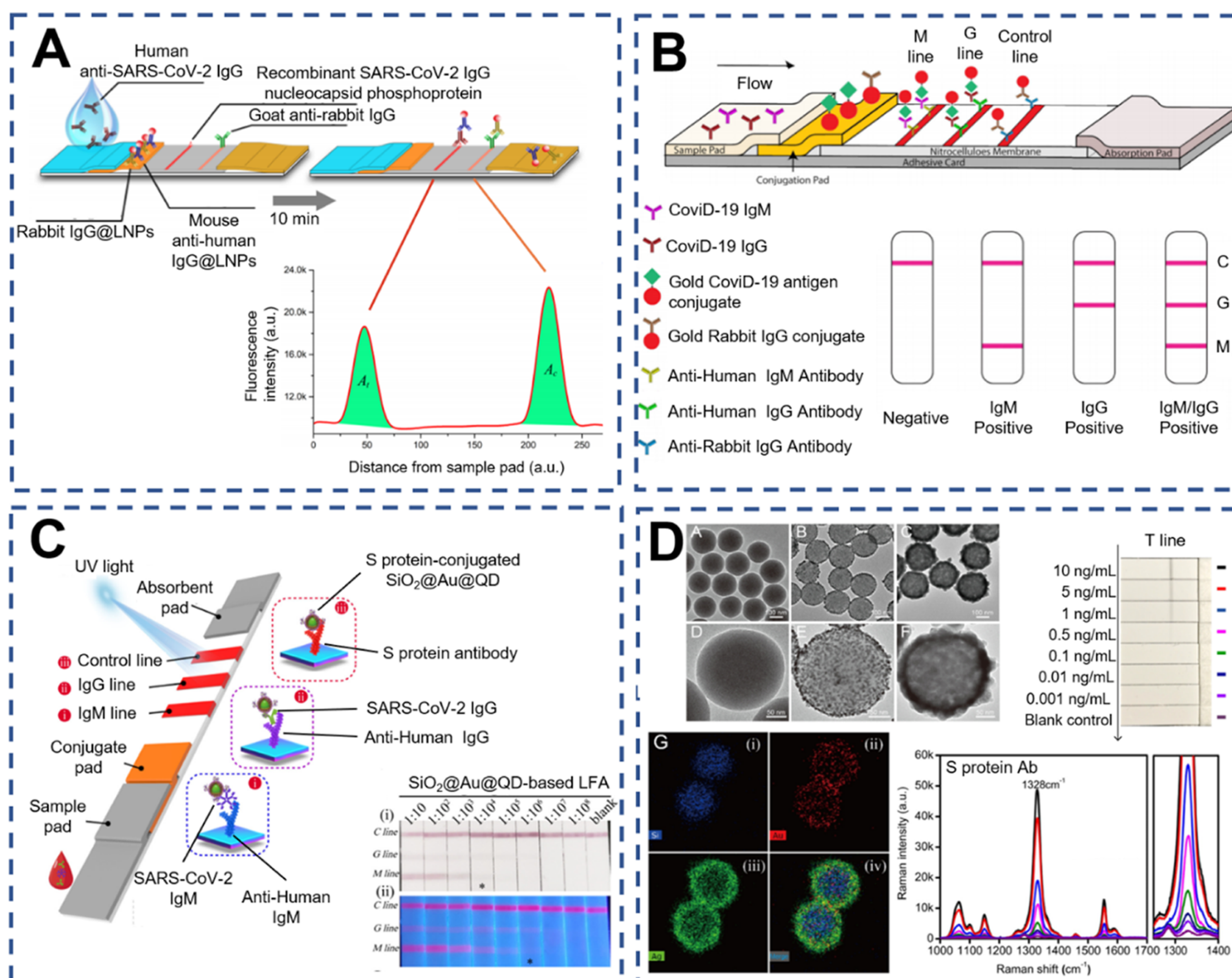

Figure 5. LFA test strip systems for detection of IgG and/or IGM antibodies. (A) Anti-nucleocapsid antigen IgG antibody detection employing lanthanide-doped polystyrene nanoparticles for highly sensitive luminescence signaling. Adapted with permission from ref 130. Copyright 2020 American Chemical Society. (B) Simultaneous detection of IgG and IgM antibodies using conjugated AuNPs-based immunoassay. Adapted with permission from ref 132. Copyright 2020 Wiley. (C) Simultaneous IgG and IgM antibodies against spike antigen, employing an alternative optical transduction method with SiO_2@Au@QD for fluorescence immunocomplex formation signaling. Adapted with permission from ref 133 Copyright 2020 American Chemical Society. (D) SERS-based LFA system for highly-sensitive anti-spike antigen IgG and IgM antibodies, employing SiO_2@Ag reporter for immunocomplex signal enhancement, down to pg/mL levels. Adapted with permission from ref 135. Copyright 2020 Elsevier.

novel hypothesis for SERS-based COVID-19 diagnosis based on the integration of a three-dimensional printed microfluidic platform with Ag-functionalized cellulose.[118] This platform is envisioned by the authors to be used with body fluids such as tears, saliva, nasal, and throat swabs, without requiring any sample pre-treatment. The combination of size dependent trapping of SARS-CoV-2 virus present in body fluids, facilitated by the microfluidic device with a paper-based SERS substrate, is expected to provide an efficient pre-concentration of the virus in the sample. Low LODs were obtained in a simple and fast detection scheme, opening the possibility for more sustainable and cost-effective SERS platforms for SARS-CoV-2 detection. More recently, Marques *et al.* reported a SERS platform based on a nanocellulose substrate, obtained from *nata de coco* cubes, with in-situ grown gold nanoparticles, assisted by microwave irradiation. With this platform, preliminary results on the label-free screening of S protein were reported, through the identification of characteristic bands of proteins.[119]

Besides optical-based detection methods, electrochemical sensing modalities have also been proposed for SARS-CoV-2 antigen detection. A paper-based electrochemical biosensor was presented by Yakoh *et al.*, reporting a label-free method for S protein antigen detection.[120] Using a chromatography paper substrate, an origami platform was developed for printing carbon-based electrodes, followed by SARS-CoV-2 IgM immobilization at the graphene-oxide modified working electrode, for antigen binding. The working principle of this biosensor was based on the inhibition of current flow through the electrodes, upon binding of the S antigen at the electrode surface, using $[Fe(CN)_6^{3-/4-}]$ as a redox probe. Higher antigen levels would cause current decrease upon the formation of immunocomplexes with immobilized antibodies, which hinders the probe's redox process on the electrode surface. Antigen levels were correlated with current within a linear range of 1−1000 ng/mL, resulting in a low LOD of 0.11 ng/mL. A similar approach was proposed by Ehsan *et al.*, using screen printing for electrode patterning, a graphene/carbon hybrid ink for the working electrode, carbon ink for the

counter, and Ag/AgCl for reference electrode fabrication.[121] The modification of the electrode was performed by an alternative method to the recurrent EDC/NHS covalent binding method, using PBASE for immobilization of anti-spike S1 unit IgG antibody. The developed electrochemical sensor was used to quantify the RBD in nasopharyngeal samples, with exceptional analytical parameters, with a limit of quantification of 0.25 fg/mL and a wide linear sensitivity range up to 1 μg/mL. This shows the great capability of paper-based electrochemical sensors to present very sensitive and specific performances, with LODs below the ones presented by traditional analytical methods such as ELISA.

3. PAPER-BASED DIAGNOSTICS FOR SARS-COV-2 IMMUNOGENIC RESPONSE DETECTION

Upon the interaction of SARS-CoV-2 with the human organism, there is an immunogenic response toward viral antigens that varies during the stages of viral infection, from the asymptomatic phase into the onset of symptoms and convalescence.[122] Immunological response monitoring against COVID-19 involves identification and quantification of specific antibodies for SARS-CoV-2, mostly immunoglobulins IgG, IgM, and IgA. At a given moment, which depends on each individual, the immunological response against the infection of SARS-CoV-2 is triggered and leads to increased levels of IgM (and IgA in saliva) and IgG.[122,123] Specific IgG and IgM have been detected in serum from 0 to 8 days of infection, by complex laboratory methods,[124] supporting that immunoglobulin levels change with each individual and within time. In a recent study, 50 and 81% of patients were positive for IgM and IgG, respectively, when first screened, having positive responses increased to 81 and 100% five days after the first measurement.[125] In general, the changing levels of immunoglobulins through time are very relevant from a clinical perspective because they can provide information about how patients handle the infection and when immunity was acquired after negative testing to the virus. The main antibodies circulating against SARS-CoV-2 target S or N proteins, and the dynamic progression of their levels change in patients at different disease stages. It has been found that the levels of N antibodies are higher in patients with a need for intensive care treatment, meaning that knowing the ratio of N and S antibodies may turn out an excellent tool for prognosis of disease outcomes.[126] Thus, serological detection of antibodies has been an important route in the control and study of this pandemic because it may serve as a diagnostic tool for present and past infections,[127] as well as aiding to better control the population immunity, especially now that vaccination is underway in multiple countries.

LFA test strips appear as an option for immunogenic response monitoring because they can provide an expedite diagnosis, with a low-cost visual transduction strategy in points of need, combining the principles of thin-layer chromatography and immune recognition reactions. Some research groups have explored this technique in the detection of IgG and IgM antibodies against SARS-CoV-2, using standard approaches such as gold-nanoparticle conjugation with the specific SARS-CoV-2 antigens, but also by using new approaches using alternative reporter particles and different optical transduction mechanisms, such as SERS or fluorescence. A novel method based on solid-phase LFA was explored by Wen *et al.*, by using the SARS-CoV-2 nucleocapsid protein for capturing the antibodies and anti-human IgG with colloidal AuNPs for analyte recognition.[128] Thus, in this experiment, indirect antigen−antibody interactions were demonstrated to form an antigen-antibody-AuNPs-mAbs complex for detection of IgG antibody against SARS-CoV-2 virus. In summary, this group optimized and validated a PoC test with high sensitivity (69.1%, with some false negative cases) and outstanding specificity of 100%. With a similar approach, the same group developed a LFA based on colloidal AuNPs-based lateral flow (AuNP-LF) for detection of the IgM antibody against the SARS-CoV-2 N protein.[129] In this study, a series of parameters were optimized, including the concentration of SARS-CoV-2 nucleoprotein, AuNP−(anti-human IgM) conjugation and BSA blocking concentration. This test was performed on specific SARS-CoV-2 IgM found in the serum of infected patients, requiring only 10 to 20 μL of sample, and leading to a sensitivity of 100% and a specificity of 93.3%, performed with a small assay time of 15−20 min.

Another LFA was developed by Chen *et al.*, using lanthanide-doped polystyrene NPs (LNPs) to detect anti-SARS-CoV-2 IgG in human serum,[130] as shown in Figure 5A. LNPs exhibit unique optical properties, such as a long luminescence lifetime (up to several milliseconds), sharp emission peaks, and up conversion luminescence, over the wide range of wavelengths, from near-infrared to visible.[131] This group used recombinant SARS-CoV-2 nucleocapsid phosphoprotein immobilized in the test line, to detect anti-SARS-CoV-2 IgG in human serum. The control line had rabbit IgG@LNPs immobilized. The method was validated with 51 healthy donor samples, 12 suspicious negative samples, and seven positive samples confirmed by RT-PCR. One of the negative samples was considered positive for SARS-CoV-2 IgG, while the results of the other samples were consistent with those obtained by RT-PCR. Based on clinical testing data, the authors concluded that there was no statistically significant difference between the results obtained by this method and those obtained by RT-PCR, also showing the possibility of past infection identification.

The combined detection of anti-SARS-CoV-2 IgG−IgM antibody by LFA has also been reported. Li *et al.* developed LFAs using AuNPs to achieve fast colorimetric outputs, taking up to 15 min, with a sensitivity of 88.66% and a specificity of 90.63%.[132] The approach taken is represented in Figure 5B and was validated by testing on 397 clinical positive and 128 clinical negative patient blood samples. In another approach, Wang *et al.* developed a colorimetric-fluorescent dual-mode lateral flow immunoassay biosensor,[133] allowing for rapid, sensitive, and simultaneous detection of SARSCoV-2-specific IgM and IgG in human serum (Figure 5C). The immunocomplexes were formed upon conjugation of S protein with SiO_2@Au@QDs nanobeads. Quantum dots (QDs) are widely used as fluorescence labels in LFAs, to improve sensitivity and quantification feasibility, due to their excellent optical properties, including quantifiable fluorescence intensity, broad excitation, and high light stability. In this work, fluorescence intensities of IgM−IgG lines were proportional to the concentrations of the target antibodies in clinical specimens. LODs for IgM and IgG were of 0.9 and 1.2 ng/mL, respectively, which could be easily read *via* a portable fluorescent instrument and used for SARS-CoV-2 IgM/IgG quantitative analysis.

SERS has also been envisioned as a very attractive alternative for optical transduction in serological antibody detection, especially for application in LFA test strips,[134] toward PoC

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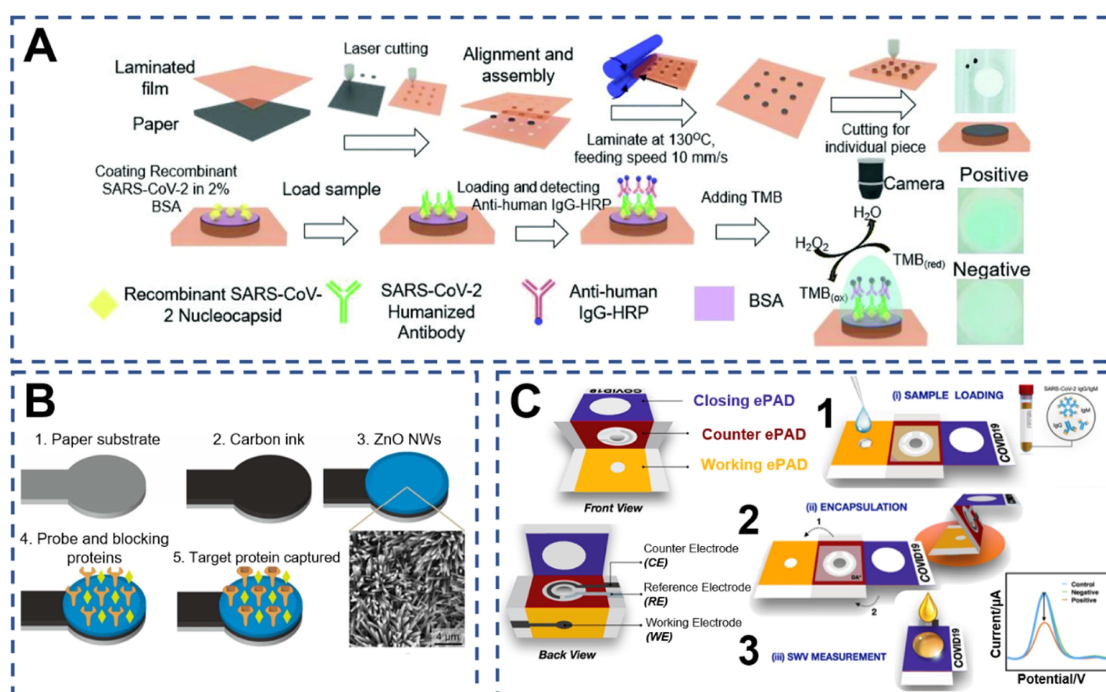

Figure 6. Alternative paper-based assay formats for anti-SARS-CoV-2 antibody detection. (A) Fabrication and preparation process of ELISA paper-based device for SARS-CoV-2 humanized antibody. Adapted with permission from ref 136. Copyright 2020 Royal Society of Chemistry. (B) Paper-based electrode fabrication for electrochemical impedance spectroscopy detection of anti-spike antigen IgG antibodies. Adapted with permission from ref 138. Copyright 2020 Elsevier. (C) Origami-style, paper-based electrochemical biosensor for IgG and IgM anti-spike antigen antibodies detection and quantification. Adapted with permission from ref 120. Copyright 2021 Elsevier.

quantitative, highly sensitive, and specific antibody sensing. Such work has been presented by Liu *et al.*,[135] where SiO_2@Ag Raman tags (SiO_2 core and Ag shell particles) were conjugated with S antigen, applied simultaneously as the capture element for IgG and IgM antibodies and as the Raman reporters, which were responsible for transducing the immune recognition reaction (Figure 5D). Upon binding of IgG and IgM antibodies in the test lines, characteristic Raman spectra and bands were identified and correlated with antibody levels. An extraordinarily low LOD of 1 pg/mL was reported, which was about 800 times better than those of conventional AuNPs-based immunoassays by LFA. Furthermore, the analysis of clinical samples revealed accuracy and specificity values of 100%, confirming the promising features of SERS-based techniques in paper-based substrates for highly accessible, quantitative diagnosis, pending its pairing with portable Raman readers.

Conventional antibody detection techniques were also successfully translated for anti-SARS-CoV-2 antibody detection in paper platforms, such as enzyme-linked immunosorbent assays (ELISA). Kasetsirikul *et al.* performed an ELISA for SARS-CoV-2 IgG antibody detection in human serum,[136] with a LOD of 9 ng/μL (0.112 IU/mL). The fabrication and preparation process of this paper-based device used a Whatman chromatography filter paper, cut in 5 mm circles and aligned between two laminate films coated with recombinant SARS-CoV-2 antigen, to capture the SARS-CoV-2 humanized antibody in the sample. Subsequently, a horseradish peroxidase/TMB system (HRP/TMB) was used for colorimetric readout of the biorecognition event (Figure 6A). Additionally, quantitative assessment was possible by digital image processing, performed with a numerical computing software. The present study achieved a simple and inexpensive colorimetric paper-based assay, with a total test time of 30 min. The sensitivity of the assay was determined with known SARS-CoV-2 humanized antibody concentrations (1 to 100 ng/μL), attaining an increasing blue color intensity for higher antibody concentrations.

To improve the functionality of such paper-based approaches, ELISA assays were also paired with paper microfluidics that allow for multiplex antibody identification and quantification from a single patient serum sample. This was performed by Gong and co-workers, using a chromatographic paper substrate and wax printing fabrication, that not only allows for the performance of all the necessary ELISA assay steps but also for the collection and sampling of serum samples in an instrument-free approach.[137] This device could detect circulating SARS-CoV-2 RBD-specific IgA, IgM, and IgG with very good detection sensitivity of 99.7%, improving on traditional antibody detection methods, also with good detection capability for detection in early infection cases.

Electrochemical paper-based analytical devices (ePAD) have also been reported for the selective detection of SARS-CoV-2 antibodies (both IgG and IgM), combining the sensitivity of the electroanalytical methods with the inherent bioselectivity of the biological component. In this context, Li *et al.* reported a paper-based gold-hybridized zinc oxide nanowires (ZnO NWs) biosensor,[138] in which the key component was the working electrode (WE). This WE was prepared in a five step-workflow, as follows: (1) cutting a paper piece into the shape of electrodes; (2) printing a layer of carbon ink to the paper piece for conductivity; (3) growing ZnO NWs on the carbon ink; (4) immobilizing the probe and blocking proteins on the surface of the WE; (5) capturing and measuring of the target protein. The combination of ZnO NWs with AuNPs provided not only improved film conductivity and better sensitivity but

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ACS Omega 2021, 6, 29268−29290

Table 2. Levels and Clinical Scenarios of Biomarkers Related to COVID-19 Progression

biomarker	normal physiological levels	COVID-19 levels	clinical scenario	reference
interleukins (IL-6, IL-8, IL10) other cytokines	low pg/mL	usually elevated levels (especially IL-10)	severe disease progression and dysregulation of inflammatory response	149
D-Dimer	<0.5 μg/mL	increase up to four-fold (above 2 μg/mL)	indicative of poorer prognosis in patients and progression to severe infection	150
C-reactive protein	<0.3 mg/mL	above ten-fold increase when compared to normal levels, to hundreds of ng/mL	elevated levels reflect evolution of pneumonia and disease severity	151
procalcitonin	0.15 ng/mL	>0.5 ng/mL	predicter of bacterial co-infection and assessment of disease severity	152
ferritin	10−250 ng/mL	in the high hundreds of ng/mL (>800 ng/mL)	more elevated levels as severity progresses, promoting stronger inflammatory response	153
lactate dehydrogenase	140−280 U/L	elevated , up to double the normal reference range	predicter of infection severity	154

also bestowed chemisorptive properties due to the gold-thiol group interactions leading to an increased stability. This biosensor could differentiate concentrations of IgG antibodies against SARS-CoV-2 spike glycoprotein S1 unit in less than 30 min, using impedimetric signal changes (Figure 6B).

Yakoh *et al.* developed another ePAD, offering a rapid and more sensitive detection of SARS-CoV-2 antibodies.[120] This platform was based on a three-electrode system, printed in an origami-styled configuration, with electrodes placed in distinct paper sheets (working ePAD, counter ePAD and closing ePAD) that are folded into the final device configuration (Figure 6C). Notably, this configuration could minimize direct contact with biohazardous fluid and avoid exposure to the environment. The WE was printed in the working ePAD, followed by immobilization of S protein RBD on the hydrophilic paper, embedded with graphene oxide (GO) by EDC/NHS chemistry. For electrochemical detection, a solution of the redox probe $[Fe(CN)_6]^{3-/4-}$ was applied to the closing ePAD. The signal detected was translated the kinetics of the redox reaction, depending on the immunocomplex formation between the captured immunoglobulins and the immobilized SARS-CoV-2 S protein. Improved analytical performance was achieved for both IgG and IgM detection, with LODs of 0.96 and 0.14 ng/mL, respectively, and a linear range between 1 and 1000 ng/mL. The analytical results obtained also compared well with the commercial standard ELISA. Electrochemical methods have shown higher sensitivity and lower limits of detection for antibody identification, however with the drawback of requiring additional equipment for signal acquisition and readout, with LFAs showing less complexity of use and lower cost.

4. PAPER-BASED DEVICES FOR COVID-19 ASSOCIATED INFLAMMATORY RESPONSE AND DISEASE SEVERITY MONITORING

After the first contact with epithelial respiratory cells, a localized and timely well-coordinated immune response presents the first line of physiological defense against SARS-CoV-2 infection.[139] Similar to other cytopathic viruses, SARS-CoV-2 infection induces cellular death and injury in airway epithelial cells through diverse processes involving pyroptosis.[140] This viral-mediated cell death causes the release of various damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), which are supposed to be recognized by pattern-recognition receptors (PRRs) from endothelial cells, dendritic cells, and alveolar macrophages. Some examples of that receptors include Toll-like receptors (TLRs), nucleotide-binding oligomerization-domain (NOD-like) receptors (NLR), C-type lectin receptors (CLRs), active protein kinase (PKR), and RIG-I-like helicase.[139] Overall, these signaling pathways foster an increased secretion of proinflammatory cytokines and chemokines, such as type II interferon (IFNγ), IL-6, interferon gamma-induced protein 10 (IP-10), and monocyte chemoattractant protein 1 (MCP1), which are responsible for subsequent pulmonary recruitment of more immune cells, including macrophages and dendritic cells.[122] Fortunately, in most COVID-19 patients, the combination of the innate immune response of the initial cytokine release and activation of the antiviral interferon response, followed by adaptive immune-cells recruitment, results in successful SARS-CoV-2 clearance from the lungs. However, as extensively reported, the viral infection can progress to severe disease, due to a deregulated immune response. So, it is of great relevance to develop rapid tests, aiming to monitor the response of the innate and adaptive immune systems against SARS-CoV-2, to better fine-tune the more effective therapeutic strategies to be adopted under these different pathophysiological conditions.

Taking this into account, several meta-analyses and published literature reported the existence of a direct relationship between inflammatory markers and SARS-CoV-2 viral infection evolution and severity.[141−144] Several inflammatory biomarkers have been associated with these outcomes, such as interleukine-6 (IL-6), interleukine-8 (IL-8), interleukine-10 (IL-10), interleukine-1β (IL-1β), C-reactive proteins (CRP), procalcitonin, ferritin, and lactate dehydrogenase (LHD), among others (Table 2).[141,145−148] Therefore, monitoring these biomarkers became inevitable, accounting their relevant role in COVID-19 management, and this is favorably performed by involving paper-based biosensors.

4.1. Interleukin 6.

IL-6 is a multifunctional cytokine involved in several immune processes (immunomodulation, hematopoiesis, and inflammation) and helps in the maturation of B cells and T cell differentiation.[155] According to the literature, high levels of IL-6 are associated with severe COVID-19 patients, as it is a crucial mediator of respiratory failure and multiorgan dysfunction.[156,157] Some reports in the literature have introduced interesting paper-based methodologies for detecting this relevant biomarker.

Cellulose-regenerated membranes were applied for selective, immune-affinity binding of IL-6 by Militano *et al.*[158] These membranes were covalently modified with protein G to allow for an oriented anti-IL-6 antibody immobilization, capturing

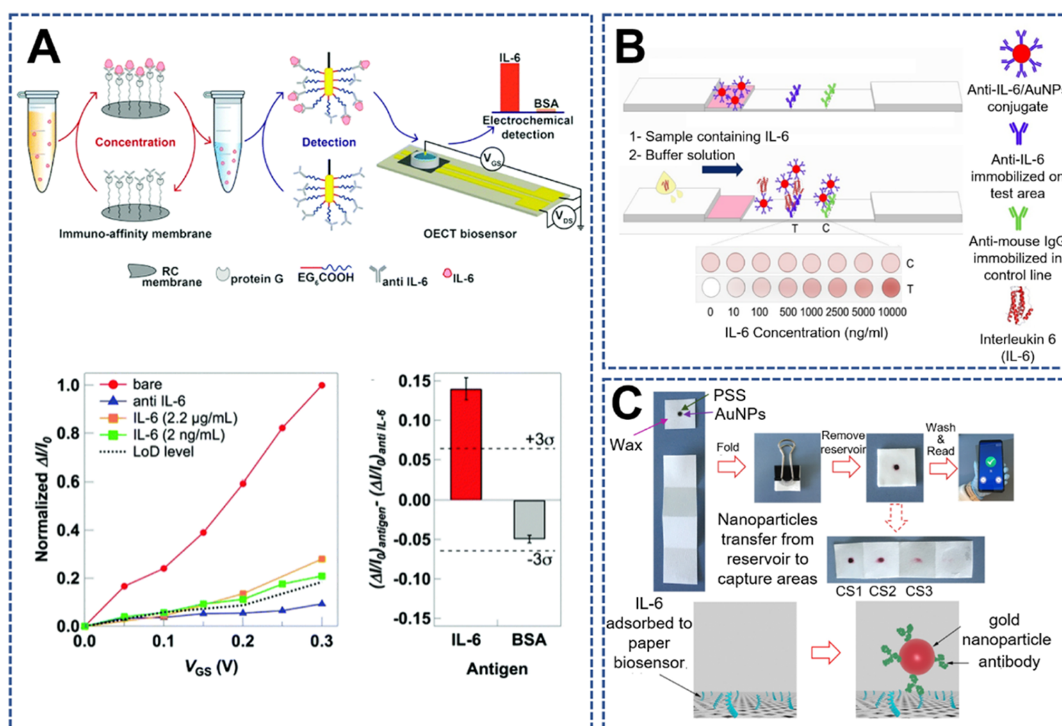

Figure 7. Paper-based biosensors for intereukin-6 detection. (A) Regenerated cellulose membranes used for concentration of the analyte applied towards sensitive electrochemical detection. Adapted with permission from ref 159. Copyright 2018 Royal Society of Chemistry. (B) LFA system for IL-6 detection at bedside, using gold-based immunoassay. Adapted from ref 160. Copyright 2020 Maples. (C) Paper-based device using a folding approach targeting IL-6 in COVID-19 patients for disease progression monitoring. Adapted with permission from ref 163. Copyright 2021 Elsevier.

after IL-6 with an exceptional efficiency. A LOD of 31 pg/ml was obtained, which corresponded to a capturing efficiency of about 91%. The authors also demonstrated the possibility of reusing such membranes, by using a cross-linking step with glutaraldehyde. Under this condition, the acidic desorption of the immune complexes allows the liberation of IL-6, and its detection by SDS-PAGE and ELISA, opening the possibility of incorporating such membranes in other bioassays and devices. Such an example has been the integration of electrochemical detection methodologies with organic electrochemical transistors (OECT), tested by Gentili and co-workers.[159] The goal of the cellulose membrane was to increase the concentration of IL-6 on the sensing electrodes, thereby improving the device response to antigen−antibody binding in a gate electrode produced with gold nanowires (Figure 7A). The combination of regenerated cellulose membranes with OECT improved the LOD, down to 220 pg/mL. Such approaches are remarkably interesting for analyte concentration steps, for application in various assays, where the target analyte concentrations may be below the necessary levels for direct measurement.

LFAs have also been used for the detection of this inflammatory biomarker. Sene *et al.* proposed an LFA method with colorimetric detection by using an anti-IL-6 antibody conjugated AuNPs.[160] The operation of this device is schematically represented in Figure 7B with a LOD of 0.38 ng/mL and a linear range between 1.25 and 9000 ng/mL, through the reading of color signal intensity at the test site, obtained in less than 20 min. Another immunoassay using the LFA format for targeting IL-6 was introduced by Ericksson *et al.*, aiming to improve the boundaries of conventional LFAs, such as the difficulties in controlling the flow rate and requirements for large volumes of antibodies.[161] Manipulating both width and geometry of a nitrocellulose test strip, sample input and output angles were optimized, resulting in enhanced analytical performance, with a 10-fold improvement in sensitivity when compared with the conventional flow, using only 10% of the volume of antibody typically immobilized on the test strip. The linear range was observed between 0.1 and 10 ng/mL, with a LOD of 29 pg/mL. Another report was presented by Borse and Srivastava, substituting the transduction element for cadmium telluride quantum dots (CdTe QDs), using the same double antibody sandwich techniques for IL-6 and CRP detection.[162] These high luminescence QDs offered sensitive and quantitative detection using a portable fluorescence strip reader developed by the authors, with a remarkably low LOD of 0.9 pg/mL and a linear detection range of 1−1000 pg/mL.

Recently, targeting IL-6 in COVID-19 patient samples has been reported to aid in disease management in a decentralized fashion. With this purpose, other paper biosensor formats were developed for IL-6 quantification (Figure 7C). Jaume *et al.* reported a paper biosensor for the detection of this cytokine, employing a chromatographic paper in the development of an origami-style device, paired with antibody decorated NPs for colorimetric signal generation.[163] Blood or respiratory samples were introduced in the device, followed by folding of a reservoir containing the colorimetric transducers. Immunocomplex formation at the paper substrate was transduced into red-colored spots that showed increasing hue values for increasing concentrations of IL-6. A color intensity cut-off was established to differentiate the sample from healthy donors and COVID-19 patients, with as low as 10 pg/mL IL-6. The same research group had also previously presented a similar assay targeting IL-6 as a sepsis marker,[164] optimizing the

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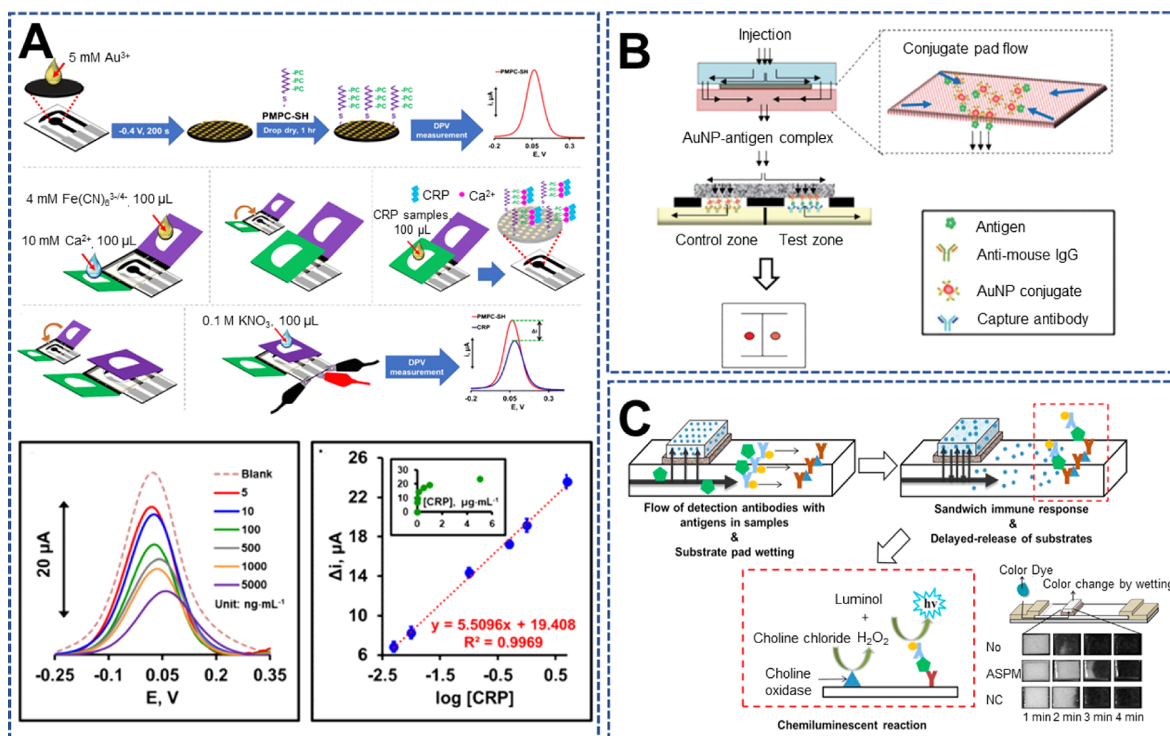

Figure 8. Paper-based biosensors for C-reactive protein detection. (A) Paper-based electrochemical biosensor using AuNPs modified electrodes, using the affinity of CRP to Ca^{2+} for binding of the analyte and subsequent current transduction. Adapted with permission from ref 167. Copyright 2019 Springer Nature. (B) Vertical flow system for AuNPs-based immunoassay, replacing conventional horizontal flow. Adapted with permission from ref 170. Copyright 2013 Royal Society of Chemistry. (C) LFA system using an asymmetric polysulfone membrane for delayed release, paired with chemiluminescence signaling for improved sensitivity. Adapted with permission from ref 172. Copyright 2014 Elsevier.

immobilization of capture antibody at the paper surface and antibody conjugation to NPs. This work employed a smartphone-based imaging processing approach for quantifying the color intensity, reporting a LOD as low as 0.1 pg/mL. These approaches showed promising features for the development of decentralized biosensing techniques that can be adapted for other important cytokines, by changing the target specific capture antibody, thereby aiding in clinical decision-making.

4.2. C-Reactive Protein. The C-reactive protein is a nonspecific pentameric protein produced by liver cells after stimulation of infection or inflammation.[165] In healthy adults, the levels of CRP are below 0.3 mg/mL, however, in COVID-19 patients, these levels are greatly increased and can be associated with more severe outcomes.[151] Several alternative paper-based biosensors, promoting more dynamic linear ranges, low LODs, and portability have been presented for CRP detection, to complement traditional ELISA used for the detection of this biomarker.

Some examples of ePADs have been presented in the literature, such as the origami paper-based analytical device for electrochemical immunoassay, proposed by Boonkaew *et al.*[166] Based on EIS readings, the device was fabricated by printing wax on a Whatman grade 1 paper substrate, followed by patterning a 3-electrode system using conductive, carbon-based ink by screen printing. The carbon ink was used to produce both the WE and counter electrode (CE), with an Ag/AgCl reference electrode (RE). The WE was modified with graphene, followed by AuNPs electrodeposition and functionalization with cysteine, for antibody binding. After all modifications, the introduction and capture of CRP to the surface of then electrodes increased the impedance, between 0.05 and 100 μg/mL, which corresponded to a LOD of 15 ng/mL. However, this work presented a long incubation time, of 50 min, for antigen−antibody binding. The same research group also presented a label-free detection approach,[167] substituting antibodies by electrodeposited AuNPs with thiol-terminated poly (2-methacryloyloxyethylphosphorylcholine) and Ca^{2+} ions, for CRP binding. Differential pulse voltammetry (DPV) readings showed decreasing currents after CRP binding to the bioreceptor for 60 min, with a linear trend between 5 and 5000 ng/mL and LOD of 1.6 ng/mL (Figure 8A). Another electrochemical biosensor was proposed by Cao *et al.*, using two different approaches with nano-ZnO/CuO and nano-ZnO nitrocellulose membranes for immobilizing anti-CRP antibodies. Both approaches were compared in terms of analytical performance by EIS and showed very low LOD values, equal to 27 pg/mL for nano-ZnO-based nitrocellulose membrane biosensors or 16 pg/mL for nanoZnO/CuO-based sensors.

Several optical sensing methods using paper substrates have also been proposed. Kim *et al.* developed a sensor that used metal clad leaky waveguide material coated with nitrocellulose for a direct immobilization of anti-CRP on the surface.[168] The results showed a linear range between 0.1 and 10 μg/mL. Oh *et al.* developed a hook effect-free (hook-effect being the decrease in signal intensity for high antigen concentrations) immunochromatographic assay for detecting CRP in serum samples, using a modified architecture for flow assays in nitrocellulose strips.[169] This technique involved immobilized capture antibodies and AuNPs conjugated antibodies, resulting in a wide linear range response, between 119 ng/mL and 100 μg/mL,

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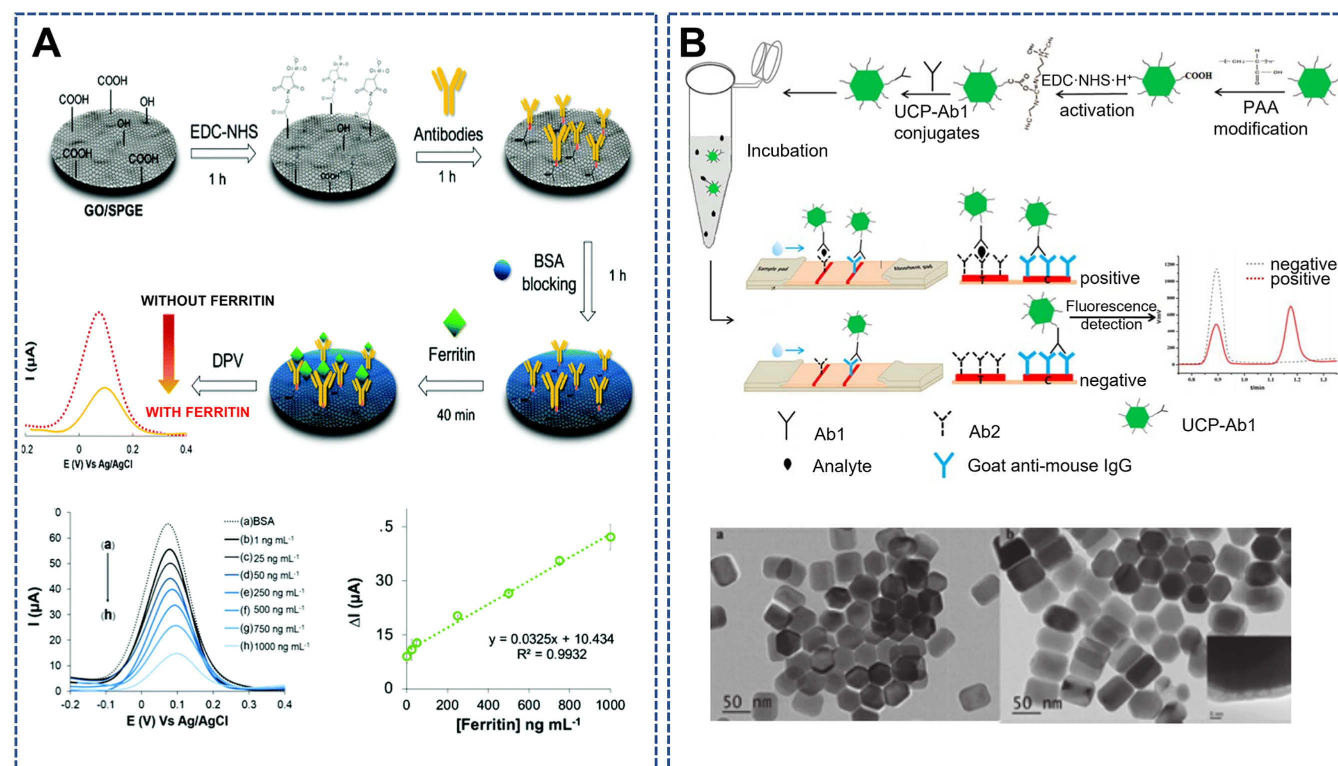

Figure 9. Ferritin and PCT paper-based detection approaches. (A) Electrochemical detection of ferritin using paper-based electrodes using immunocomplex formation causing alterations in current signals correlated to the presence of ferritin. Adapted with permission from ref 177. Copyright 2020 Royal Society of Chemistry. (B) LFA system for PCT detection, using UCP conjugated with anti-PCT antibodies, for sensitive fluorescence detection. Adapted with permission from ref 182. Copyright 2017 Wiley.

with a LOD of 43 ng/mL. Another work developed by the same research group used a vertical flow immunoassay strategy.[170] In this, the device components were assembled vertically, including sample and conjugate pad, flow-through holes film and nitrocellulose membrane. The color signal was obtained for a sandwich antigen−antibody reaction with conjugated AuNPs, displaying a linear range between 0.01 and 10 μg/mL, with a low assay time of 2 min. The authors compared their method with the conventional LFA. The results demonstrated a gradual increase of the signal, with extended sensitivity for the vertical assay (Figure 8B). Another LFA-based immunoassay was presented by Shen *et al.*, using water-soluble $CuInZn_xS_{2+x}/ZnS$ core/shell nanocrystals as fluorescent labels.[171] Their conjugation with target capture antibodies was successfully established, producing test strips of good analytical performance, with a low LOD of 1 ng/mL. Another approach using an alternative optical transduction mechanism consisted in the application of enzyme immunoassay in LFA format, made by Joung *et al.*.[172] In their work, HRP-labeled antibodies were used to produce a chemiluminescent reaction, using an asymmetric polysulfone membrane (Figure 8C). This membrane was introduced between the nitrocellulose membrane and the substrate pad to render a delayed release of reagents. The results of this LFA showed a chemiluminescent signal with a linear response range between 1 and 10000 ng/mL and a LOD of 1.05 ng/mL.

An alternative paper-based analytical device was reported by Lin *et al.*, who developed a paper-based diagnostic device for blood CRP monitoring, based on the agglutination of blood serum upon CRP reaction with Ca^{2+} ion.[173] This reaction resulted in different flow lengths, through the microfluidic channels in the device. The possibility of using alternative transduction methods with cellulosic materials was also explored by Zhang and Rojas, using quartz crystal microgravimetry (QCM) modified with ultrathin films of carboxylated cellulose nanofibers.[174] Using protein A for an oriented immobilization of anti-CRP antibodies, the resulting method displayed a linear range of 1 to 100 μg/mL and LOD of 0.1 μg/mL.

4.3. Ferritin. Ferritin is a metalloprotein responsible for storing iron in the liver. It takes part in immune dysregulation processes inducing systemic inflammation, contributing to the cytokine storm. According to the WHO, normal levels in healthy adults are in the range of 15−200 ng/mL (in women) and 30−300 ng/mL (in men).[175] For severe COVID-19 cases, higher levels of ferritin around 1000 ng/mL have been reported, showing that this is a relevant biomarker related to the prognosis of patients.[176] Not many reports on paper biosensors have been presented in the literature targeting hyperferritinemia, making this a hot field to explore and develop paper sensors that can fill this gap. Recently, Boonkaew *et al.* developed a graphene-modified paper-based electrochemical sensor for the detection of ferritin, using a Whatman filter paper grade 1[177] and the overall approach shown in Figure 9A. Patterned hydrophobic and hydrophilic areas were established by printed three-electrode systems, using inkjet printing. The WE and CE were printed with graphene ink on the hydrophilic area of the substrate and the RE was printed with Ag/AgCl ink. The WE was modified with a graphene oxide solution by drop-casting on the surface, followed by surface activation with EDC/NHS chemistry, for covalent binding of anti-ferritin antibodies. The electro-

chemical behavior of the sensor was evaluated by DPV, with results showing a decreasing current that was associated with the formation of immunocomplexes at the electrode surface, due to the presence of ferritin bound to the biorecognition element. The analytical performance for ferritin detection showed a linear range between 1.0 and 1000 ng/mL, with a LOD 0.19 ng/mL.

4.4. Procalcitonin. Procalcitonin (PCT) is a precursor of the calcitonin hormone and is a biomarker for lower respiratory tract bacterial infections and sepsis.[178] Normal levels of PCT are below 0.15 ng/mL,[179] and elevated levels may indicate severe infection outcomes in COVID-19 patients and is positively correlated with mortality in critically ill patients.[180]

Some methods for PCT detection used electrochemical and fluorescence systems on paper substrates. Recently, Boonkaew *et al.* developed an electrochemical paper-based analytical device coupled with a label-free immunoassay for the simultaneous determination of three biomarkers in serum samples, CRP, cTnI, and PCT.[181] In this work, a graphene-modified stencil-printed carbon electrode (G-SPCE) was modified with graphene oxide, for subsequent oxidation and covalent binding of target specific antibodies by carbodiimide chemistry (Figure 9A). The current response of the modified electrodes to the presence of each biomarker was quantified by square wave voltammetry, translated by decreasing currents against increasing concentrations of the target analyte, caused by the formation of immunocomplexes. The selectivity of this sensor was evaluated with several interfering species and the results revealed a great selectivity of the immunosensors. The multiplex electrochemical immunosensor showed a linear range from 1 to 1000 ng/mL for CRP, 0.001 to 250 ng/mL for cTnI, and 0.0005 to 250 ng/mL for PCT, with LODs of 0.38 ng/mL for CRP, 0.16 pg/mL for cTnI and 0.27 pg/mL for PCT. This method offered high selectivity, reproducibility, and stability, for up to 1 month. Although its target was for cardiovascular disease monitoring, these principles can be applied for monitoring these important metabolites in COVID-19 prognosis management.

For optical detection of PCT using LFA test strips, Lei *et al.* introduced the application of upconverting NPs (UCP) technology for immunochromatographic assay (UPT-ICA) for rapid and quantitative detection of PCT,[182] as shown in Figure 9B. In this work, the authors compared and evaluated PCT levels in blood with UCP-Ab1 conjugates, before and after freeze-drying. The results presented improved sensitivity of freeze-dried-UCP-Ab1, with lower background and stability. The analytical performance provided a linear response ranging from 0.5 to 100 ng/mL, with a low LOD of 0.19 ng/mL.

5. OUTLOOK AND FUTURE PERSPECTIVES

The urgent need to control COVID-19 pandemic has identified that efficient, rapid, and low-cost diagnostic methods that may be promptly available in a global basis, are essential. Huge efforts have been made to this end, including the development of novel approaches for molecular diagnosis, immunoassays, and other sensing methods, using various transduction mechanisms, both for laboratorial and PoC settings. Through the research output presented in this review, it is seen that paper and other cellulose-based materials have confirmed their potential for application in multiple biosensing schemes, primarily targeting cost-effective, accessible, portable and more sustainable alternatives that may complement SARS-CoV-2 identification and diagnosis, while also being able to aid in COVID-19 management in infected patients. Although gold-standard RT-PCR molecular diagnosis for SARS-CoV-2 has been established and is aiding in effective diagnosis, gaps can still be filled in order to achieve widespread testing in multiple epidemiological situations. In parallel with laboratorial techniques and other biosensor development approaches, paper-based devices can be a viable option to reach this goal. For example, NAATs diagnosis have appeared as novel, high-performance detection methods, also paired with other genetic editing tools, creating new trends and approaches for SARS-CoV-2 detection, but also opening the possibility for application in other current or future viruses. By translating such approaches into paper-based devices, different tasks (sample mixing, amplification, readout and other complementary steps) can be performed in the same platform, bringing the possibility for more simplified and highly reliable diagnostic techniques to be performed in a PoC setting. Furthermore, as shown in this review, such methods complemented other conventional methods, including immunoassay approaches or affinity-based biosensing, using different transduction mechanisms. Concurrently, the availability of other paper-based testing methods has risen, not only in research and development settings but also in commercial forms, mainly through LFA rapid testing for antigen identification and serological antibody detection, portraying the potentialities of cellulose materials to be readily applied in current and future pandemic situations, as a rapid aid in the diagnostic needs of the future. Some considerations are still needed when developing such sensing alternatives, mainly their analytical performance and clinical validation that must have to follow strict regulations and requirements to be used both in clinical setting and self-testing. Overall, these paper-based tools may find many applications in epidemiological settings, including inoculation and immunity assessment of populations exposed to the virus or that have already taken recently developed vaccines.

Importantly, paper-based sensing systems may undergo a wide range of other applications in COVID-19 management, much beyond human analysis. One promising application in this regard is their use in wastewater monitoring, aiming to explore epidemiologic data that may act as an early warning indicator of viral spread. Indeed, many reports now confirm that the dissemination of COVID-19 in wastewaters is a global concern.[183] Sewage systems contain potentially infected excreta from wastewaters collected in homes and urban public facilities, for which their contents may evidence abnormal viral spread, even before this is detected in clinical settings. Environmental contamination outcoming form these biologically contaminated wastes may also take place under specific conditions, which raises relevant safety concerns to public health and to other living species, as well as to the overall environment. Although there are no current studies for this purpose involving paper-based biosensors BioMark@UC and i3N/CENIMAT, research groups are implementing joint studies to develop innovative LFA and electrochemical systems, under the context of the Eco2Covid project. Until now, obtained results are very promising, and soon the sensing platforms will be validated in wastewater samples. Studies to develop other innovative paper-based platforms for the inexpensive and fast detection of antibodies against SARS-CoV-2 are also taking place in combined efforts of the same

https://doi.org/10.1021/acsomega.1c04012
ACS Omega 2021, 6, 29268−29290

research groups and BioMark@ISEP, through the implementation of TecniCov project.

Overall, paper-based sensing systems can be used for simple screening or accurate quantification of samples, using paper test-strips, lateral flow systems, and electrochemical biosensors, in combination with computational tools for data collection and analysis. Another important consideration for future research in the development of paper-based sensing tools is the possibility for comprehensive quantitative sensing, enabled by portable readers and information technology tools such as functionality-embedded smartphones or portable potentiostat units with signal transmission capabilities,[184] such as near-filed communications (NFC) for signal readout. These tools enable interaction and integration with the IoT for transmission of data. In pandemic scenarios, this allows for more controlled communication of important health information, which is relevant for controlling and haltering the infection transmission, also providing the capability of integrating artificial intelligence (AI) tools and system developed for more fluent management of epidemiological situations in COVID-19.[185,186] Although many qualitative detection tools have been put to place in the SARS-CoV-2 pandemic, as previously pointed out, quantitative measuring would be of help in situations where viral load has shown to be an important factor in virus spreading and disease outcome. Paper-based platforms have shown to be very versatile and adaptable for pairing with such reading devices, bringing the new possibility of generating extremely sensitive techniques, such as specific optical (fluorescence, SERS) and electrochemical sensing methods to be translated into PoC, in multiple settings, where access to centralized healthcare may not be readily available. Moreover, the analytical features can be improved by incorporating different nanomaterials or other synthetic recognition elements, such as molecularly imprinted polymers, on biosensors. Nanomaterials such as nanozymes or metal NPs can be highly beneficial to detect SARS-CoV-2 infection or antibodies in different samples. It can contribute to decrease the limit of detection and increase its sensitivity during the recognition process. This improves the prospects for reliable self-testing, decreasing the burden on healthcare facilities that many times have been overwhelmed with the high demand for testing and treatment of infected patients.

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The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge funding through projects Eco2Covid (POCI-01-02B7-FEDER-068174) and TecniCov (POCI-01-02B7-FEDER-069745), co-funded by FEDER through COMPETE2020 and Lisboa2020. T.P., A.R.C. and A.C.M. acknowledge funding to National Foundation for Science and Technology, I.P., FCT, through their PhD grants, references DFA/BD/8606/2020, SFRH/BD/130107/2017 and SFRH/BD/115173/2016, respectively.

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