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COVID-19: A challenge for electrochemical biosensors

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A R T I C L E   I N F O

A B S T R A C T

Coronavirus disease (COVID-19) caused by SARS-CoV-2 has spread since the end of 2019 and has resulted in a pandemic with unprecedented socioeconomic consequences. This situation has created enormous demand for the improvement of current diagnostic methods and the development of new diagnostic methods for fast, low-cost and user-friendly confirmation of SARS-CoV-2 infection. This critical review focuses on viral electrochemical biosensors that are promising for the development of rapid medical COVID-19 diagnostic tools. The molecular biological properties of SARS-CoV-2 as well as currently known biochemical attributes of infection necessary for biosensor development are outlined. The advantages and drawbacks of conventional diagnostic methods, such as quantitative reverse-transcription polymerase chain reaction (qRT-PCR), are critically discussed. Electrochemical biosensors focusing on viral nucleic acid and whole viral particle detection are highlighted and discussed in detail. Finally, future perspectives on viral electrochemical biosensor development are briefly mentioned.

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1. Introduction

The new coronavirus SARS-CoV-2 first emerged in December 2019 in Wuhan, Hubei Province, China [1]. Due to its highly contagious nature, it has been rapidly spreading worldwide [2]. SARS-CoV-2 belongs to the order *Nidovirales*, family *Coronaviridae*, subfamily *Orthocoronavirinae* genus *Betacoronavirus* and subgenus *Sarbecovirus*, which also include severe acute respiratory syndrome-related coronavirus (SARS-CoV) and Middle East respiratory syndrome-related coronavirus (MERS-CoV) [3–5]. Virions of the *Coronaviridae* family are roughly spherical, 118–140 nm in diameter and notable for the large spike glycoprotein that extends from the virus envelope, which gave the name to the whole family (Fig. 1).

In 2002, SARS-CoV emerged in Guangdong Province (China) and spread to five continents through air travel routes, infecting 8098 people and causing 774 deaths. No cases of SARS-CoV have been reported worldwide since 2004. MERS-CoV causing severe lower respiratory tract infection in people emerged in the Arabian Peninsula in 2012, where it remains a major public health concern, and was exported to 27 countries, infecting almost 2500

individuals and claiming 858 lives. By the end of August 2020, more than 21.2 million confirmed COVID-19 cases had been reported by the WHO, including 761,000 deaths.

SARS-CoV-2 mortality can significantly differ depending on the geographic area. Reports show that SARS-CoV-2 is rapidly moving across countries, and genomes with new mutation hotspots are emerging [6]. Thirteen variation sites in SARS-CoV-2 open reading frame 1 (ORF1) were recently characterized, with some positions showing mutation rates up to 30% [7]. The mutagenic process of the viral genome depends on viral enzymes that replicate nucleic acids, which is affected by little or no proofreading capacity and/or post-replicative nucleic acid repair. In most viruses, RNA polymerase lacks proofreading capability, with some exceptions, such as the *Nidovirales* order (to which the *Coronavirus* genus belongs). Eight new recurrent RNA-dependent RNA polymerase (RdRp) mutations in SARS-CoV-2 were characterized by Pachetti et al., with differences in occurrence in Europe, Asia and North America. Each of them possesses a different mutation pattern, showing the contribution of RdRp to maintaining its proofreading capability [6]. Due to the aforementioned mutations, early detection and monitoring of spread and disease development by standard RT-PCR might be challenging due to possible primer mismatches with targets [8]. Hence, focusing on conserved regions of the viral genome and multiple virus gene targets were recommended to minimize the probability of false-negative results [9].
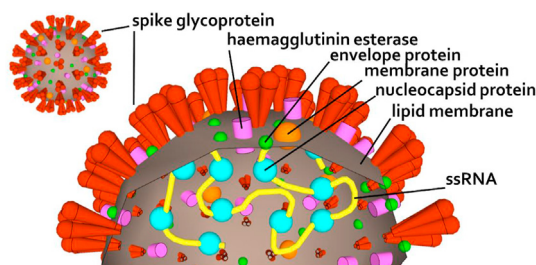
Fig. 1. Schematic of the SARS-CoV-2 structure showing the surface structure (left) and cross-section (right).

Among quarantine, staying home and social distancing measures, rapid testing represents one of the cornerstones in slowing virus transmission and helps to understand SARS-CoV-2 epidemiology. Currently, qRT-PCR, antigen and serological (IgM and IgG antibodies) tests are used for COVID-19 diagnosis. PCR tests are considered the gold standard of COVID-19 testing, and together with antigen tests, PCR tests are able to determine active infection in patients who shed the virus. Although qRT-PCR possesses adequate sensitivity to determine early infection, false-negative or false-positive results in the range of tens of percent have been reported by several authors [10,11]. Although false-positive results have economic consequences of quarantine and contact tracing, in the case of false-negative results, infected people can spread the virus even without symptoms. Among the virus mutations mentioned above, Tahamran and Ardebili suggested that sample type, sampling and viral load kinetics can influence the results of PCR tests [12]. Serological tests determine blood anti-SARS-COV-2 IgM/IgG status and provide information about recent SARS-CoV-2 exposure (IgM and IgG positive) and past infection (IgM negative and IgG positive); however, IgM-positive and IgG-negative status can denote an active infectious state. Notably, no test can provide 100% sensitivity (the likelihood that the test will be positive for infected persons) or specificity (the likelihood that the test will be negative for non-infected persons), and all test results must be carefully interpreted with other clinical features, such as computed tomography images [13]. Woloshin et al. highlighted the urgent need for the determination of clinical and analytical test performance and for reference standards for measuring the sensitivity of SARS-CoV-2 tests in asymptomatic persons [14].

Electrochemical biosensors represent alternative approaches to detect viral nucleic acids or viral antigens and can contribute to the development of point-of care (POC) COVID-19 testing. Employment of "point-of-care-testing" (POCT) methods is worthwhile due to the prospect of immediate determination of the patient's negative/positive status at the site of testing within minutes or 1 h. Many POCT methods could be potentially useful for such purposes, but if the qRT-PCR cost, specificity and sensitivity requirements need to be fulfilled, the choice begins to be markedly limited.

The aim of this conceptual review is to discuss and conceptualize the use of electrochemical (bio)sensors for designing coronavirus POC analytical devices, which might be beneficial to maintain sensitivity, selectivity and costs at acceptable levels.

2. Analytical hallmarks of the viral target

2.1. Detection window

Many patients experiencing SARS-CoV-2 infection are not even hospitalized and have asymptomatic development. This fact causes many of these patients to not undergo qRT-PCR testing after interacting with their general practitioner. The critical point is that infectious persons are not determined and may still contribute to the worsening of the epidemiological situation. Fig. 2 describes the detection window when anti-SARS-CoV-2 antibodies cannot be detected and the different stages of immune response to infection (Fig. 2). In the first to second week after infection, nucleic acid-based POCT can represent an alternative to conventional qRT-PCR. However, after 10−14 days, when seroconversion occurs, antibody-based POCT can play an important role.

The main factor influencing the practitioner's choice of analytical method is the cost of the qRT-PCR procedure, which consists of 3 general parts: i) acquiring the sample (personal time and collection material), ii) transport to a certified laboratory and iii) testing in the certified laboratory (pre-analytical phase costs such as biosafety level certification, qRT-PCR kit costs, and administrative costs). Next, the POCT can benefit from decreased expenses to only i) acquire the sample, ii) kit costs, and iii) send the data via a network (e.g., smart city platforms) to the nearest epidemiologic authority.

If captured within the correct timeframe after disease onset, SARS-CoV-2-specific antibody responses can be helpful for the rapid determination of active infections and past infections. Hence, the added value of serological testing is the determination of past infection, where qRT-PCR fails. Seroconversion occurs from approximately 10 days after symptom onset, but the exact timing of IgM (green line) and IgG (red line) appearance is currently unclear. The IgG antibody titres rise from day 10 onwards to reach a peak whose height is likely to be influenced, on a case by case basis, by disease severity and virus load (difference between the solid red, and dashed red and blue lines). Currently, the exact level of antibody protection against reinfection (black dotted line), the duration of the total humoral immune response above this level and the rate of decline of mild or severe infection-induced antibody levels are not known. Similarly, the proportion of infected individuals who do not mount a protective immune response (green line) has not been determined [15].

2.2. SARS-CoV-2 PCR detection

The order *Nidovirales* contains positive-sense, single-stranded RNA (ssRNA) genomes that are capped and polyadenylated. The *Nidovirales* order contains enveloped viruses with the largest RNA genomes among currently known RNA viruses in the range of 12.7−33.5 kb, which can infect a variety of vertebrate and invertebrate hosts [16,17]. Unique among positive-sense ssRNA viruses, large nidoviruses encode a 3′-to-5′ exoribonuclease (ExoN) that is implicated in controlling RNA replication fidelity. Nidovirus genomes are typically organized into many ORFs occupying over 90% of the genome. The products of these regions predominantly
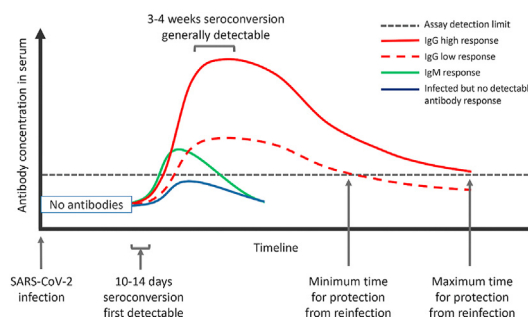
Fig. 2. A schematic representation of the immune response following SARS-CoV-2 infection (adapted from Ref. [15]).

control genome expression, replication, virus assembly and dissemination [16,18].

Most of the molecular diagnostics being developed for the diagnosis of COVID-19 infections involve qRT-PCR test methods similar to those developed for the diagnosis of SARS-CoV. Coronaviruses contain molecular targets within their genome that can be used for PCR assays, including genes of structural proteins, the envelope spike glycoprotein (S protein), envelope protein (E protein), membrane protein (M protein), helicase (Hel), and nucleocapsid protein (N protein), as well as genes required for viral replication, such as RdRp, haemagglutinin esterase (HE), and open reading frames ORF1a and ORF1b [19—21].

Centers for Disease Control and Prevention (CDC) recommends two N protein targets (N1 and N2) while the WHO recommends first line screening with the E gene assay followed by a confirmatory assay using the RdRp gene [22,23]. Many laboratories have developed assays independently of the CDC or WHO. Chan et al. developed three novel qRT-PCR assays targeting the RdRp/Hel, S, and N genes of SARS-CoV-2 [24]. Chu et al. described two 1-step qRT-PCR assays to detect two different regions (ORF1b and N) of the viral genome separately [23]. Two molecular targets (E and RdRp) were selected for testing in the study by Corman et al. [20]. The nucleocapsid and ORF1ab genes were chosen in other studies [24—26]. Despite a considerable number of recent studies there has been no indication that any one of these sequence regions used offers a significant advantage for clinical diagnostic testing. Ideal design would include at least one conserved region as well as a specific region to mitigate the effects of genetic drift, especially as the virus evolves within new populations [22].

3. Design of coronavirus biosensors

Undoubtedly, electrochemical biosensing of viruses is challenging and ambitious. However, electrochemistry provides several well-known advances for biosensor design, such as inherent high sensitivity, relatively low-cost sensors and equipment, user-friendly manipulation, fast analysis, and suitability for miniaturization, hence creating POC devices. Current state-of-the-art electrochemical biosensors and related technologies are critically discussed in subsequent paragraphs.

3.1. Recognition element

SARS-CoV-2 possesses several suitable targets for biological recognition elements. First, viral RNA is one. Viral nucleic acids are definitely the ideal target since they are suitable for early stage-detection when the IgM and IgG immune responses are still too low. Myriad studies have reported hybridization electrochemical biosensors aimed at viral nucleic acids and have claimed their reliable detection [27—32]. However, most of these studies have used synthetic oligonucleotides as analytes, which are often just slightly longer than the corresponding probes. Such biosensors are suitable for the detection of PCR products where the quantity of amplicons is relatively high and the only interferents are polymerases, primers and nucleoside triphosphates, but they can hardly be used for the detection of viral genomic nucleic acids in real biological samples. Notably, regarding genetic complexity and genome size, the *Coronaviridae* family is the largest RNA virus identified to date (29.7 kb) [33].

Antibodies are accepted as the gold standard for biorecognition, and they are broadly used in medical diagnostic and therapeutic practice. Antibody affinity towards antigens (reaching the nanomolar range in the case of good antibodies) has resulted in various electrochemical biosensors ranging from enzyme-amplified ELISA-like to label-free formats. However, there are numerous challenges that antibody-based research faces. Many researchers have highlighted the insufficient characterization and low quality of antibodies used in biomedical research, causing their poor reliability by binding to a plethora of irrelevant antigens [34,35]. Engineering recombinant antibodies provided extensive possibilities for modifications with tags, labels or reporters and the production or creation of antibody fragments. In contrast to heterogeneous polyclonal antibodies, monoclonal and recombinant antibodies are homogeneous molecules that bind to single antigen epitopes. This attribute makes them more sensitive to pH, salt concentration or temperature change. Especially in the field of biosensors, where multiple washing steps are inherent parts of working procedures, such conditions can induce uncertainties in the results, which further highlights the need for antibody validation [36]. Recent advances in this field were reviewed by Park [37].

Regarding antibodies focusing on whole viral particles, the most problematic issue is their long-term reliability; the viral genome changes under immune system pressure and can result in epitope changes. Approximately 15 amino acids (discontinuous in the protein primary sequence) of the epitope come into spatial contact with the antibody paratope [38]. Of these 15 amino acids, 5 strongly influence antibody-antigen interactions, and their point mutation can decrease the relative binding constant by two or three orders of magnitude [39]. Currently, researchers focus on antibodies against the SARS-CoV-2 S protein of the viral envelope. By binding to the S protein, these antibodies prevent viral particles from interacting with cellular angiotensin-converting enzyme 2 (ACE2) receptors; hence, they are able to neutralize (neutralizing antibodies, nAbs) infection and should help with vaccine design [40]. Such antibodies are suitable for the development of whole viral particle biosensors since they focus on highly exposed parts of viral antigens [41]. Although there is overall structural homology between SARS-CoV and SARS-CoV-2 S proteins, Wrapp et al. reported that none of three SARS-CoV S protein receptor-binding domain (RBD) binding monoclonal antibodies bind to the SARS-CoV-2 S protein [42]. Although several commercially available antibodies against the S protein and other SARS-CoV-2 structural proteins (E, M and N protein) are available, their specificity against individual serotypes of the virus has not been determined. Notably, slow and long epidemics allow selection pressure to drift further and possibly create novel mutated antigens [43]. A well-reported case of an S protein point mutation (D614G), which appeared in early April 2020 and as of the end of the year has become the dominant variant in the pandemic, has been reported [44,45]. Hence the performance of biorecognition elements of viral biosensors and POC devices needs to be frequently re-evaluated.

Aptamers, short ssRNA or DNA molecules, are able to bind target molecules with high affinity and selectivity. Many aptamers focusing on inorganic molecules, proteins, cells and whole viral particles have been reported. Aptamer degradation can play an important role in the field of biosensors [40]. In particular, RNA aptamers are prone to degradation by RNases. The ubiquitous occurrence of RNases due to their secretion by human skin and air-dispersed microorganisms can lead to surface contamination. In addition, the presence of RNases in real samples, such as body fluids such as tears, saliva or sweat, can hamper the performance of aptasensors [46]. Hence, RNA aptamers need to be modified to protect against nuclease activity [47].

The development of antiviral aptamers is a difficult task. If whole viral particles are considered, membrane proteins are targeted first. However, full-length membrane protein purification is a rather complex issue. Natural protein structures cannot be mimicked by recombinant extracellular parts of proteins that are subject to vector-dependent post-translational modification and do not oligomerize. Cell-SELEX (systematic evolution of ligands by

exponential enrichment), which uses whole cells instead of target proteins, was developed to overcome these drawbacks. Recently, an advanced SELEX method (Viro-SELEX) suitable for the development of membrane protein aptamers was developed [48]. It uses enveloped viruses, such as the baculovirus expression system, to incorporate membrane proteins, such as influenza haemagglutinin (HA), into the surrogate viral envelope and for whole viral particle aptamer enrichment through SELEX [49]. Recently, Song et al. reported two SARS-CoV-2 DNA aptamers targeting the RBD of the S protein [50]. The dissociation constants (K_d) of CoV2-RBD-1 (51-base) and CoV2-RBD-4 (67-base) were 5.8 and 19.9 nM, respectively. Based on previously selected SARS-CoV aptamers, Chen et al. designed an aptamer for the SARS-CoV-2 N protein, which was shown to be a promising early-stage diagnostic marker in serum [51,52].

There are other biorecognition elements that have been proven to be beneficial for electrochemical viral biosensor development. This advance can be demonstrated using the typical case of influenza. Sialic acid (SA), covering a broad range of neuraminic acid derivatives, is a part of host cell surface glycoproteins and glycolipids that are recognized by the viral glycoprotein HA and mediate endocytosis and infection. Notably, SA serves as a receptor for many other viruses [53]. Recognition of various SA derivatives and conformations by HA genotypes determines influenza virus transmission. Immobilized SA can serve as a recognition element of viral biosensors. For example, Hiroguchi et al. used various SA molecules immobilized on electrodes to distinguish between human and avian influenza A [54]. Analogous to the case for influenza, ACE2 which mediates SARS-CoV-2 entry into cells, can serve for biosensor biorecognition purposes [55].

In recent years, mimicking the immunological interaction between antibodies and antigens with molecular imprinting strategies has been trending [56]. Although the technology was originally designed for small molecules, molecular imprinting has extended towards supramolecular complexes including viruses. Still facing many challenges, such as virus size, fragility or instability in organic solvents, the most recent publications in the field address these challenges. For example, Gast et al. imprinted hexon protein (the most abundant and accessible protein of human adenovirus type 5 capsid) and reported high selectivity of this solution towards entire viral particles [57]. Successful imprinting of the SARS-CoV-2 structure on a polymer was demonstrated in a reprint by Parisi et al. [58]. They fabricated "monoclonal-type" plastic antibodies (nanoparticles using MIP technology) specific for the RBD of the SARS-CoV-2 S protein with no cross reactivity towards the RBD of the SARS-CoV S protein. Possession of the mentioned antibodies was also declared by the commercial company MIP Diagnostics Ltd. (Bedford, UK).

3.2. Sensitivity challenges of viral biosensors

The main challenge of viral electrochemical biosensors is their sensitivity. SARS-CoV-2 shares epidemiological attributes with the previous epidemic agents SARS-CoV and MERS-CoV. In the case of many infections, specific IgM antibodies can be detected after 3 days of non-immunodeficient patient infection. IgM represents the first line of organism defence against viral infections and hence can indicate recent exposure to viruses. In contrast, IgG can be detected with a delay of several days. Although the detailed immune system response to SARS-CoV-2 virus has still not been described, we can expect similar serological responses, such as in the case of SARS-CoV. Anti-SARS-CoV IgM and IgG antibodies were reported to be detectable after 3–6 and 8 days, respectively [59]. Zhou et al. showed that anti-SARS-CoV-2 IgM and IgG peak at 9 days and within a second week after disease onset, respectively [2]. It is

important to note that the immune response of individual patients is variable, making serological testing prone to errors. For example, Long et al. showed that plateaued IgG and IgM levels can vary widely (by more than 20-fold), IgG and IgM titres differ between severe and non-severe patients, seroconversion shows distinct patterns, and even hospitalization with IgG- and IgM-negative status is rare [51]. Taking into account the fact that symptoms can occur even after 24 days (3 days median) and occurrence of patients without any symptoms, tracking virus spread is a difficult task, and combined IgG/IgM-based-rapid tests are beneficial but do not ensure reliable SARS-CoV-2 confirmation in the early stage after onset [52]. The field of serological rapid diagnostics of SARS-CoV-2 infection is dominated by lateral flow test strips, which were reviewed in detail by Quesada et al. [60]. Currently, commercial companies are selling corresponding rapid diagnostic kits or serological ELISA kits enabling high throughput and multiple formats of assays (electrochemiluminescence assay, enzyme immunoassay or fluorescent immunoassay). Sinawang et al. demonstrated that integration of lateral flow technology and electrochemical detection is beneficial and can substantially decrease demands for operators' manual operations [61]. However, such an approach can hardly compete with user-friendly optical evaluation of common lateral-flow strips.

In our opinion, early detection of viral infection is the field where electrochemistry can prove its potential. However, we will discuss the obstacles of such an approach first. The important topic is definitely analytical parameters, which biosensors need to fulfil. The viral RNA loads are as similarly variable as antibodies. They are not constant during the infection and change by several orders of magnitude. Pan et al. reported that the viral load of throat swabs and sputum peaked approximately 5–6 days after symptom onset and reached 10^4 - 10^7 copies per ml during this time [62]. However, He et al. observed the highest viral loads in throat swabs at the time of symptom onset and concluded that infectiousness peaked on or before onset [63]. Recent findings declare that viral loads of asymptomatic and symptomatic patients are similar, suggesting that identification of patients with no or mild symptoms is possible [64]. By simple calculation, the target concentrations of SARS-CoV-2 biosensors should be in the range from tens of attomolar to tens of femtomolar. Although the viral load of COVID-19 patients will be more specified in the future, it is evident that most of the currently published viral hybridization biosensors do not meet these analytical parameter demands. Hence we can conclude that various advanced signal amplification strategies need to be adopted.

3.3. Sample treatment

Antibodies are produced by B cell lymphocytes and are secreted into the bloodstream hence free antibodies can be found within bodily fluids such as blood, serum, plasma, saliva and tears. However, the sensing of viral nucleic acids is completely different. As declared several times, real sample preparation represents a critical bottleneck of biosensor translation from research laboratories to clinical practice or even personal analytical devices [65]. The sample treatment procedure starts with obtaining the sample. Most POC tests use swabs for sampling. During the COVID-19 pandemic, nasopharyngeal swabs, oropharyngeal swabs and sputum are common clinical samples. No discernible differences were observed between naso- and oropharyngeal swabs showing viral load on the order of magnitude of 10^5 RNA copies per swab even in the early stages of disease [66]. As Zasada et al. reported, the role of swab properties should not be underestimated [67]. They showed that swab material was related to the adsorbed volumes of the specimen and elution buffers to the amount of recovered nucleic acid. To detect virus infection by the interaction of the

electrochemical biosensor surface with viral RNA/DNA, nucleic acids need to be released and recovered from viral particles by disruption of the viral capsid or optionally the viral envelope. Here, several approaches can be adopted. Occasionally, simple heating (45−100 °C) of the sample can be sufficient for some applications. In the case of possible nucleases within the sample, the addition of ethylenediamine-tetraacetic acid (EDTA) or glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA) is recommended to chelate divalent and trivalent cations required as co-factors for several nucleases. However, neither EDTA nor EGTA inactivates RNases hence alternative approaches such as the use of diethyl pyrocarbonate (DEPC)-treated or polyvinyl sulfonic acid-treated solutions, have been developed [68,69]. Although such chemical treatment of samples can interfere with subsequent PCR amplification, hybridization of nucleic acids and application of electrochemical biosensors should not be influenced. Chaotropic salts (sodium iodide or guanidinium thiocyanate) or detergents (sodium dodecyl sulfate, SDS) are used in combination with heating to disrupt capsids by denaturing and minimizing inter- and intra-protein hydrophobic interactions. In addition, guanidinium thiocyanate is beneficial and hence inhibits RNases.

The biology of SARS-CoV-2 simplifies further sample treatment. SARS-CoV-2 belongs to the group of positive-sense ssRNA viruses. First, nucleoproteins are not strongly bound to RNA and do not need to be removed before analysis. Second, most DNA viruses and some RNA viruses possess double-stranded nucleic acids. In these cases, biorecognition of nucleic acids by hybridization targets with probes is not possible because even after denaturation, long single strands possess higher affinity towards each other than short oligonucleotide probes. However, the possibility of sequence-specific targeting of dsDNA via triplex-creating or duplex/triplex invasion with protein nucleic acids, locked nucleic acids or bridged nucleic acids has been reported [63,70,71]. Notably, long sequences of viral ssRNA/ssDNA are prone to create thermodynamically stable but flexible secondary structures caused by intramolecular base pairing, in which groups of nucleotides are not accessible for complementary strands and can prevent target nucleic acids from hybridizing to probes. ssRNAs of exact secondary structures are functional products that play regulatory roles in essential processes such as replication, repair, transcription, translocation and recombination. Heat denaturation of ssDNA/ssRNA is necessary before target-to-probe hybridization and hybridization at increased temperatures and salt concentrations is desired.

3.4. Practical consideration

In this chapter, we will focus on practical examples of viral infection biosensors, which, in our opinion, have demonstrated innovative and unique approaches. In this field, we have focused on only biosensors of viral nucleic acids and whole viral particles, hence representing the only possibility for early stage diagnostics and being suitable for the design of point-of-need devices (see Fig. 3).

The first study we decided to mention is on Zika virus detection demonstrated by Lynch et al. [72]. Zika virus is, as along with SARS-CoV-2, a positive-sense ssRNA virus (Fig. 3a). They used nucleic acid sequence-based amplification (NASBA) for viral RNA fragment amplification. Further, they immobilized a DNA stem loop on the electrode and used two DNA adaptor strands partially complementary to both the target viral amplicon and stem loop to perform biorecognition. One DNA adaptor is labelled with electroactive methylene blue and responsible for the target recognition (blue probe), and the other is responsible for target RNA secondary structure unwinding (green probe). In the presence of the target, a 4-way junction structure comprising an unwinded stem loop, both

DNA adaptors and target RNA is created and brings redox markers in close proximity to the electrode surface, hence providing a detectable signal. They reached biologically relevant concentrations of the target in unpurified amplicons in less than 1 h. Notably, NASBA is a highly suitable tool for ssRNA rapid diagnostic test development because no denaturation steps are required here for RNA fragment amplification, and the whole process is isothermal (approximately 40 °C). Hence, expensive thermocyclers are not necessary.

Another alternative method for isothermal nucleic acid amplification is loop-mediated isothermal amplification (LAMP). The combination of LAMP with magnetic separation and electrochemical detection of human papillomavirus DNA fragments (HPV16 and HPV18) was reported by Bartosik et al. [73]. Magnetic particles were shown to be useful in the analytical preconcentration of samples and hence are being used to enhance the sensitivity of electrochemical viral biosensors [74,75]. They mixed a LAMP-obtained solution with magnetic particles modified with a capture probe targeting the LAMP amplicon. Washed particles were subsequently incubated with an anti-digoxigenin antibody conjugated with horseradish peroxidase (HRP); hence, digoxigenin-labelled deoxyuridine triphosphate was a part of the LAMP mastermix. They utilized magnetic attraction of the construct on the surface of electrode and amperometric sensing of the HRP oxidation product (benzoquinone).

An interesting approach for various influenza A subtypes was reported by Matsubara et al. [76]. They demonstrated that biorecognition based on receptor-ligand binding can be beneficial. More precisely, they targeted influenza viral particles via membrane fusion glycoprotein HA affinity to a sialyloligosaccharide receptor-mimicking peptide. On a boron-doped diamond electrode, they showed a linear response between 3 and 400 pfu·ml^{-1}, as reported by the charge transfer resistance induced by adhered viral particles. However, the biosensor was not tested using a real sample.

A common approach for enhancing electrochemical biosensor sensitivity, which is highly desirable, is to increase the surface of working electrodes by various nanostructures or attach biorecognition elements to nanomaterials or engineered biomaterials. For example, Baek et al. attached norovirus-targeting peptides to WS$_2$ nanoflower composites with gold nanoparticles [77]. This construct was used to bind norovirus particles from real sample extracts and was drop-cast on carbon screen-printed electrodes (SPE), where the norovirus signal was recognized as an increased charge transfer resistance of electrochemical impedance spectroscopy plots (LOD 2.37 copies · ml^{-1}). An alternative approach to improve biosensor performance was used by Chavan et al. [78]. They increased the amounts of antibodies bound on the electrode by bioengineered apoferritin, which resulted in 24 protein-G units and 6 histidine tags per apoferritin molecule. Histidine tags served for apoferritin immobilization on electrodes via nickel nitrilotriacetic acid (Ni-NTA) linkers and protein-G for anti-infectious pancreatic necrosis virus antibody attachment.

An electrochemical biosensor of influenza reaching an appealing LOD of 5−10 viral particles per sample in 5 min was reported by Nidzworski et al. [79]. They targeted the influenza M protein, which forms a coat within the viral capsid (Fig. 3b). The biosensing mechanism was based on immobilization of anti-M antibody on a boron-doped diamond working electrode via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry on electrografted 4-aminobenzoic acid. The binding of the M protein on the electrode increased the charge transfer resistance against Fe(CN)$_6^{3-/4-}$. To analyse real samples, induced nasal swabs were collected in buffer containing 0.5% Triton X-100, allowing M1 protein to be released. They obtained stable
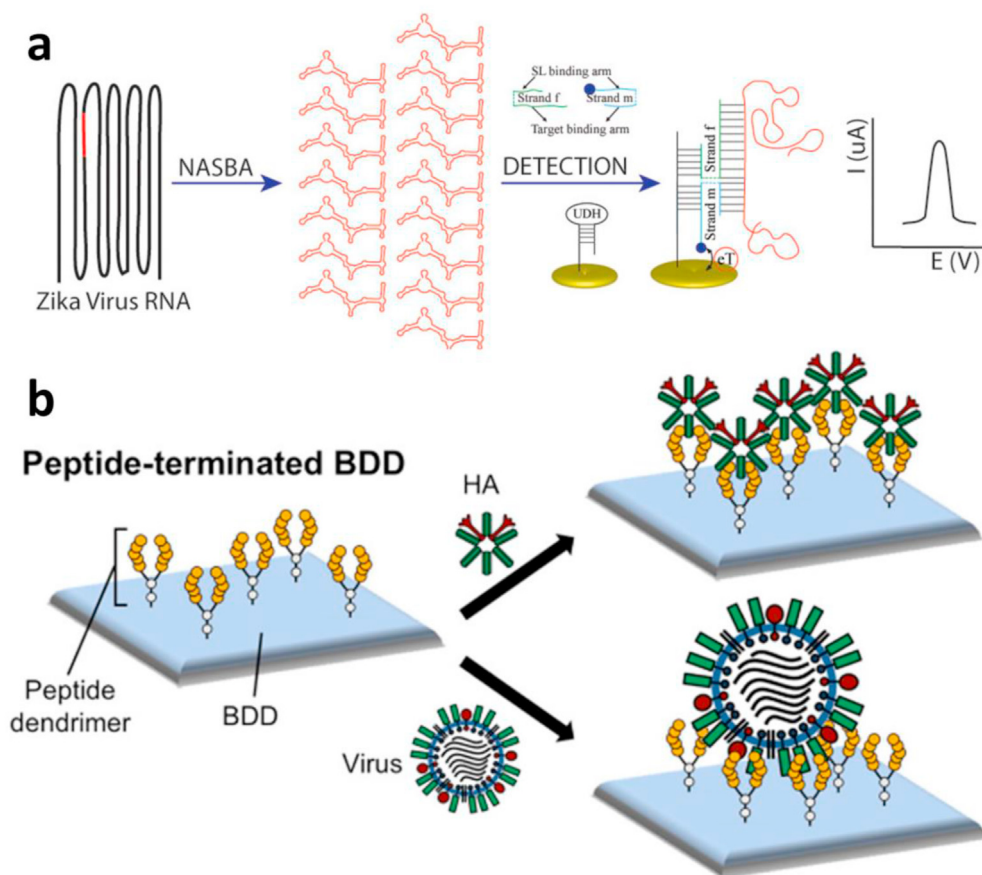
Fig. 3. Schematic representation of (a) HA and influenza virus binding to peptide-terminated boron-doped diamond (BDD) electrodes (adapted from Ref. [72]), and (b) schematic representation of HA and virus binding to peptide-terminated BDD (adapted from Ref. [79]).

impedimetric signals of electrochemical impedance spectroscopy (EIS) even in the presence of bacteria and yeast within the sample. The analytical parameters of the mentioned biosensors are summarized within Table 1.

An electrochemical immunoassay for SARS-CoV-2 was reported by Fabiani et al. [80]. They focused on the S protein and N protein and reached LODs of 19 and 8 ng.ml^{-1}, respectively. Their workflow comprised i) pre-coating of magnetic particles (not included in the

Table 1
Summary table of viral biosensors.

analyte	target type	amplification	reporter	method	LOD	linear range	electrode	time (min)	reference
zika virus	NA[a]	NASBA	methylene blue	SWV[b]	1.11 fg μl^{-1} 1.8·10^5 copies·ml^{-1}	1−75 nM	GDE[c]	60	[72]
HPV[d]	NA	LAMP	hydroquinone	AMP[e]	0.1 ng	0.1−50 ng	SPE chip	150	[73]
norovirus	NA	−	Fe [(CN)$_6$]$^{3-/4-}$	EIS[f]	2.37 copies·ml^{-1} in spiked sample; 6.21 copies·ml^{-1} in real sample	up to 10^4 copies·ml^{-1}	SPE	60	[77]
influenza	WVP[g]	−	Fe [(CN)$_6$]$^{3-/4-}$	EIS	0.33−0.91 pfu[h]	20−400 pfu	BDD electrode	−	[76]
pancreatic necrosis virus	WVP	−	Fe [(CN)$_6$]$^{3-/4-}$	EIS	2.69 TCID$_{50}$·ml^{-1i}	100−100000 TCID$_{50}$·ml^{-1}	GDE	−	[78]
influenza	WVP	−	Fe [(CN)$_6$]$^{3-/4-}$	EIS	1 fg ·ml^{-1} 5−10 WVP per sample	up to 100 fg	BDD electrode	5	[79]
SARS-CoV-2	WVP	−	1-naphthol	DPV	6.5 pfu·ml^{-1}	−	SPE	30	[80]
SARS-CoV-2	WVP	−	−	DPV	1.68·10^{-22} μg ·ml^{-1}	−	SPE	1	[81]
SARS-CoV-2	NA	−	toluidine blue	DPV	200 copies·ml^{-1}	1 pM - 10 aM	SPE	181	[82]

[a] Nucleic acid.
[b] Square wave voltammetry.
[c] Gold disc electrode.
[d] Human papillomavirus.
[e] Amperometry.
[f] Electrochemical impedance spectroscopy.
[g] Whole viral particles.
[h] Plaque forming unit.
[i] Fifty-percent tissue culture infective dose.

total analysis time), *ii)* immunoassay on magnetic particles as a solid support with a reporting antibody labelled with alkaline phosphatase (ALP), and *iii)* electrochemical analysis. The product of enzymatic cleavage of 1-naphthyl phosphate by ALP, 1-naphthol, was analysed using carbon black-modified screen-printed electrodes and differential pulse voltammetry (DPV) [83]. The report addresses several current challenges, such as total analysis time (below 30 min here), easy sampling (untreated saliva) and portability (printed sensor and portable potentiostat). An interesting sensor (no biorecognition element) for various viruses based on the differentiable fingerprint of their glycoproteins in voltammograms was reported by Hashemi et al. [81]. They benefited from the properties of graphene oxide modified with 8-hydroxyquinoline, EDC and NHS composites with gold nanostars, which have high adsorptive capability for viral S proteins due to the presence of various functional groups. Label-free DPV performed on glassy carbon electrodes modified with the composite provided peaks at different potentials for infectious bronchitis virus, avian influenza, Newcastle disease virus and even SARS-CoV-2 in untreated blood and saliva; hence, the potentials of peaks served for virus identification and the peak intensities served for virus quantification. Compared to RT-PCR they declared only 5% miss rate in the case of SARS-CoV-2 in just 1 min of analysis. A complex nanotechnological approach to the SARS-CoV-2 genome using a supersandwich-type biosensor was developed by Zhao et al. [82]. First, they selectively isolated clinical RNA samples using magnetic particles modified with thiolated capture DNA probe (CP) via affinity of thiols to gold. Second, the fabricated p-sulfocalix [8] arene-functionalized graphene was loaded with gold nanoparticles and toluidine blue (electrochemical label). To gold nanoparticles in the composite, a partially hybridized labelling probe (LP) and auxiliary probe (AP) were attached using a labelling probe thiol group. Probes were designed such that the 5′- and 3′-termini of the target sequence were complementary to the CP and LP, respectively, and the 5′- and 3′-regions of AP had complementary sequences with two different LP areas. Therefore, sequence-specific detection can be achieved by using CPs and LPs, and AP hybridizes many times with the LP to produce long concatamers, resulting in high biosensor sensitivity. The reported biosensor provided a remarkable LOD of 200 RNA copies $\cdot$ ml^{-1} in clinical specimens, and the detectable positive rate was 85.5% in confirmed patients.
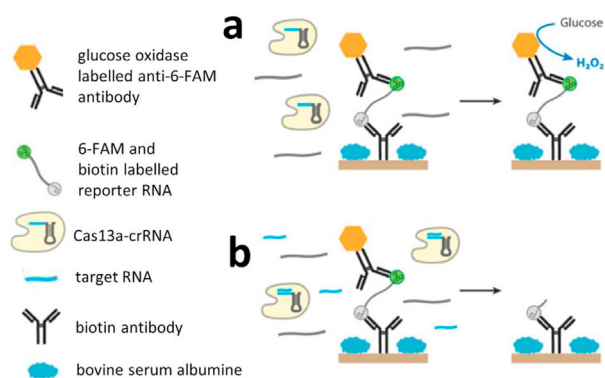
Fig. 4. Collateral cleavage-based electrochemical RNA biosensor. (a) Without the presence of the target RNA, the Cas13-crRNA complex exhibits no cleavage activity towards the reporter RNA; hence, the glucose oxidase-labelled 6-FAM antibody remains immobilized on the electrode via affinity of the biotin antibody and reporter RNA labelled with 6-FAM and biotin. Hence, glucose oxidase can mediate amperometric detection of H$_2$O$_2$. (b) However, if the target RNA is present within the sample, Cas13a-crRNA cleaves the RNA reporter, and glucose oxidase is washed out and provides no (or lower) current response (modified from Ref. [87]).

3.5. Perspectives of viral electrochemical biosensors

The versatility of the systems, which would enable us to easily detect new analytes, paves the way for new pathways in the strategy of designing such platforms. In this field, CRISPR systems in combination with bioengineered enzymes with cleavage activity and nucleic acid amplification are of great interest. Approaches such as CRISPR/Cas9 triggered isothermal exponential amplification reaction (CAS-EXARP) and NASBA-CRISPR cleavage (NASBACC) can be used to genotype pathogens and differentiate single nucleotide polymorphisms (SNPs) [84,85]. In 2016, a comprehensive study of the type VI CRISPR/Cas system demonstrated the collateral cleavage enzymatic activity of Cas13a after its target-specific binding [86]. This approach was used by Bruch et al. to develop an electrochemical nucleic acid (miRNA) biosensor (Fig. 4) [87]. Collateral cleavage-based CRISPR/Cas technology has the potential to significantly affect the field of biosensors by offering a much more rapid and precise method for ultrasensitive nucleic acid detection. Even CRISPR/Cas-based deployable POC paper devices may be possible [88,89]. Due to the great potential offered by CRISPR/Cas-based biosensing, several articles have been published as reviews, news, comments, or insights on CRISPR diagnostics since 2017 [90], which represent evidence for great potential in this field of biosensor development.

4. Conclusion

In summary, the presented review summarizes current state-of-the-art approaches to viral electrochemical biosensors, with a focus on SARS-CoV-2. We provide a brief overview of qRT-PCR and serological tests, show their advantages and drawbacks and consider them regarding advances in electrochemical tests. Individual parameters of electrochemical biosensors are critically discussed, promising works are described and we provide future perspectives of the field. We expect that the SARS-CoV-2 pandemic will accelerate the transformation of many analytical approaches from proof-of-concept status to applied technologies.

We demonstrate that electrochemistry brings several advantages among conventional SARS-CoV-2 diagnostic methods. First, it can provide solutions for portable analytical devices composed of miniaturized sensors and minipotentiostats compatible with smartphones. Fast on-site and accurate analysis helps to reduce the economic damage of infection spread and possible quarantine. Furthermore, in comparison with qRT-PCR, electrochemical biosensors can offer attractive price per analysis. Sampling and sample treatment represent critical attributes of POC devices, and even minimal manual operations mean disqualifying drawbacks. It is evident that neither qRT-PCR and serological tests, nor electrochemical SARS-CoV-2 biosensors are flawless tools for COVID-19 diagnostics, but these techniques should complement each other. As we demonstrated, electrochemical biosensors can focus either on viral nucleic acids or whole viral particles. We believe that electrochemistry can provide solutions for rapid and inexpensive POC tests, which should be the first diagnostic method a potentially infected person uses prior to clinical diagnosis. The serious socio-economic consequences of false-negative tests must be considered, and we suggest that attention should be paid to the sensitivity, selectivity and positive predictive value of published and especially commercial SARS-CoV-2 diagnostic tools.

Declaration of competing interest

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

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