Human monkeypox virus: Detection methods and perspectives for diagnostics

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A B S T R A C T

The recent outbreak of the zoonotic human caused by the monkeypox virus, in several non-endemic territories, raised concerns about the emergence of a new pandemic. Still concerned about the recent SARS-CoV-2 pan- demics, people have been gathering efforts to control the spread of the monkeypox virus. Human Monkeypox is a disease characterized mainly by the skin lesions caused, it is considered an endemic and neglected African disease that has caused several deaths throughout history. However, recently it was detected in more than 28 non-endemic countries, including the United States and European countries. Furthermore, there is still a lack of data on the extent of infection in asymptomatic people, making it a public health problem. In this aspect, rapid and large-scale testing followed by the isolation of infected individuals is one of the most effective ways of prevention known, as the monkeypox vaccine is not widely available. Thus, this review article presents an overview of recent outbreaks of monkeypox, and the detection methods currently available for the control of this disease. Also, we addressed the potential application of modern methods, which are highly attractive and effective alternatives for aiding in monkeypox diagnosis. Furthermore, we discuss the perspective of using diagnosis in regions of difficult access and poor infrastructure to public health and the control of a highly dangerous virus.

1. Introduction

Despite Human Monkeypox being considered a neglected disease until now, it was first identified in the Democratic Republic of Congo in 1970, causing numerous outbreaks in the African continent [1]. However, in May 2022 researchers found the presence of this virus in humans in the United States of America and, since then, it has been reported in more than 28 non-endemic countries and territories [2]. Although it has been considered a low pandemic potential virus, compared to COVID-19, the increase in the number of cases has been causing great concern to the World Health Organization (WHO) and healthy authorities from several countries [3].

Containment strategies of an epidemiological outbreak, as seen recently in the COVID-19 pandemic, are directly linked to prophylactic actions, such as the implementation of social withdrawal, stimulation of the immunological system by vaccination, and proper treatment of infected individuals. However, for this to be achieved it is necessary to carry out massive testing of the population. This has become one of the main global challenges within this context since many countries do not have enough infrastructure. The inefficiency of mass testing usually causes a delay in decision-making and, consequently, in the disease control measures, such as the isolation of infected people, including asymptomatic ones [4]. The extent to which the asymptomatic infection occurs is not well known, and it is not clear whether asymptomatic cases or undiagnosed infections are empowering the outbreak [5].

For these reasons stated above, access to devices capable of

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performing the early, simple, and effective diagnosis of Monkeypox is essential, aiming at containing the disease, as well as decreasing the hospitalization and mortality index [6]. Despite the disease being neglected for years, there are a significant number of scientific works aimed at monitoring it mainly by polymerase chain reaction (PCR) [7, 8]. However, it can be performed by other strategies, such as based on virus culture [9], enzyme-linked immunosorbent assay (ELISA) [10,11], loop-mediated isothermal amplification (LAMP) [12,13], recombinase polymerase amplification assay (RPA) [7,14], and clustered, regularly interspaced, short palindromic repeat, associated protein Cas (CRISPR-Cas) [15,16]. Nonetheless, in addition to the existence of commercial recombinant monoclonal antibodies for this virus, great advances have been also observed in the better understanding of the nature of the disease, such as the sequencing of some fragments of the virus [17], which enhances the possibility of developing methodologies for its monitoring.

The present review article aims to address the main characteristics of the Monkeypox virus disease, showing aspects of transmissibility and spreading, and present the main methods employed in the determination of the virus causative of this disease. Furthermore, perspectives on the development of electrochemical methods, still not explored for the detection of the Monkeypox virus, have been highlighted. Different strategies have been discussed, addressing the possible biomarkers available for the construction of electrochemical biosensors for the determination of the virus, showing the possibility to develop simple and miniaturized devices for fast analysis of the Monkeypox virus.

2. Monkeypox virus: an overview

Monkeypox is a zoonotic viral disease caused by the monkeypox virus, which is one of the species of a large double-stranded DNA family of viruses (*Poxviridae* family), belonging to the genus *Orthopoxvirus* [18, 19]. Poxvirus commonly presents nanometric (200—450 nm in length) egg- or brick-shaped particles, which have been found in biological samples of infected patients by transmission electron microscopy (TEM) (Fig. 1A and B). The virus particles consist of a complex structure, containing a core, lateral bodies, and outer membrane, covered by an outer lipoprotein envelope [20–23]. The outer surface of the virus particle is responsible for protecting the nucleic acid genome from damage, and also for recognition and interaction with host cells. On the other hand, the function of the lateral bodies is still unknown [23]. The viral double-stranded DNA (dsDNA) and core fibrils are located in the central core (Fig. 1C). Several viruses from this group are known to cause infectious diseases between humans, such as variola virus (smallpox), cowpox virus, vaccinia virus, and monkeypox virus.

Monkeypox was first observed and identified in cynomolgus monkeys in 1958, at the Statens Serum Institut – Copenhagen, Denmark. The virus had been isolated from infected monkeys, leading to the name of the virus and the disease [24]. Nevertheless, twelve years after that episode, the first human monkeypox case was reported in a child, in the Basankusu Territory, Democratic Republic of the Congo [25]. Since its discovery, sporadic cases of human monkeypox have been reported in different continents, such as Africa, North America, and Europe.

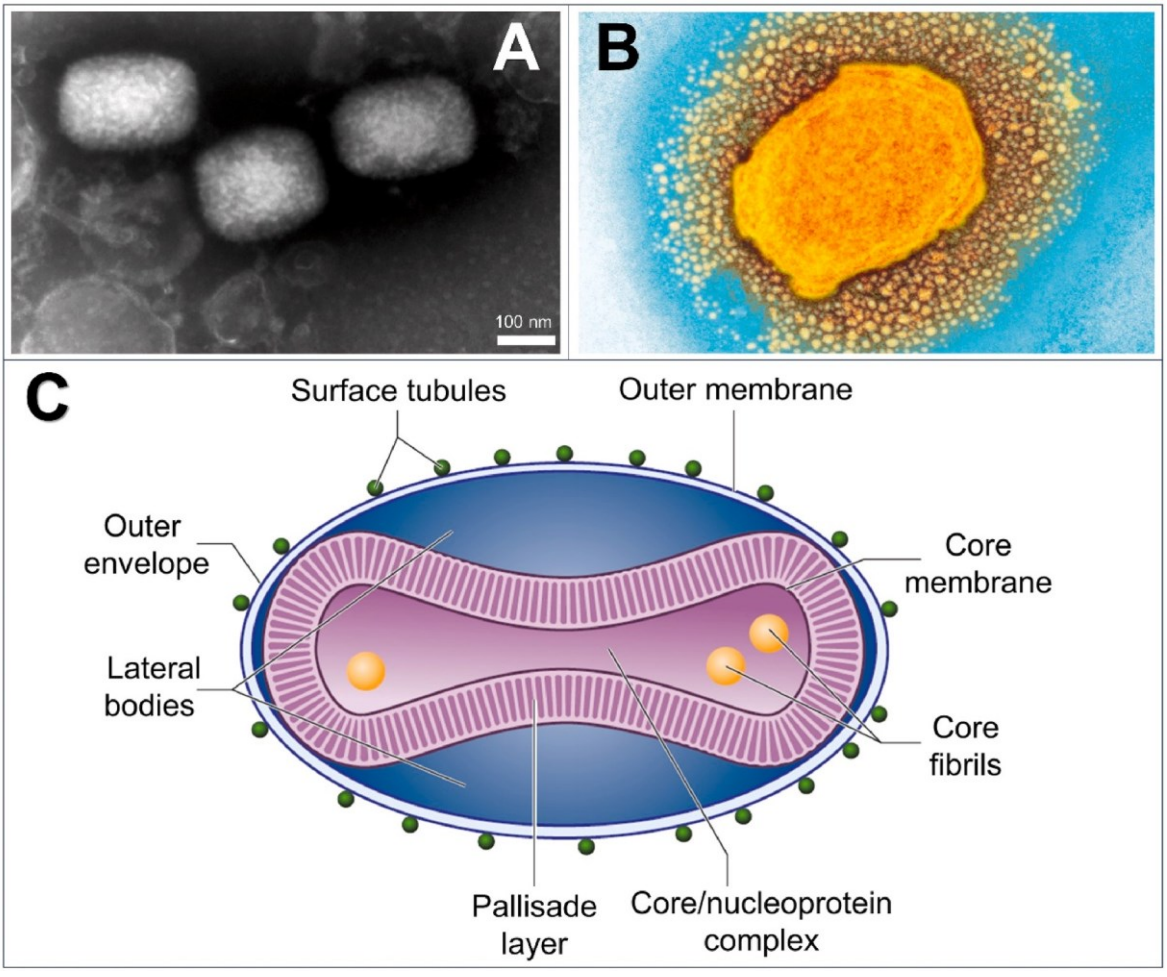

Fig. 1. Transmission electron micrographs (TEM) of monkeypox virus particles (A) in black/white with the magnification of 150,000 × and (B) coloured TEM. (C) Schematic illustration of a poxvirus particle. Adapted with permission from (A) Chmel, M. et al. Viruses, 14, 1773, 2022. Copyright (2022), MDPI [21]; (B) Kozlov, M. *Nature*, 606, 2022. Copyright (2022), Nature [22]; (C) Cann, A. J. *Principles of Molecular Virology*, 6th ed. Elsevier. Copyright (2015), Elsevier [23].

However, it is considered an endemic disease in Africa, mainly in the west and central portions of the continent [26,27].

The disease is characterized by lesions on the skin, which appear mainly on the body extremities (*i.e.*, face, legs, arms, and genital regions) during the eruptive phase, lasting 2–4 weeks [28]. In addition, distinct symptoms have been described during the invasive or early phase (0–5 days), which include fatigue, lethargy, asthenia, and fever [26]. Although the poxviruses (*i.e.*, monkeypox, chickenpox, and smallpox) are genetically distinct, these symptoms are commonly seen in pox-like illnesses, which can make them indistinguishable [28]. The possibility of infection by the monkeypox virus is considered when the patient presents fever or unexplained rash, in addition to two or more other symptoms with the first symptom, observed 21 days after exposure to the virus [28].

The life cycle of this virus occurs in the cytoplasm of infected cells by a complex route (Fig. 2A), which can be initiated by both intracellular mature or extracellular enveloped virions (IMV or EEV) by attachment, fusion, and core entry steps [29]. For the extracellular enveloped virus, its external membrane can attach to the host cell membrane. The external membrane is removed, exposing the mature virus, which fuses with the cell [30,31]. Once the virus core is released into the cytoplasm of the host cell, viral messenger RNA (mRNA) begins to be synthesized under the control of the virus (virus factory) [32]. Viral replication is characterized by three stages of viral mRNA and the synthesis of the protein (translation stage), which are assembled alongside the DNA sequences formed in the replication stage (assembly), followed by the morphogenesis of infectious particles [20,30]. In this step, the intracellular mature virion (IMV) is formed. Then, the intracellular enveloped virion (IEV) is formed by wrapping it with Golgi-derived membranes [20]. Finally, the produced virus buds from the host cell, being now called extracellular enveloped virions (EEV) [30,33,34].

3. Human contamination and transmission

Monkeypox virus can be transmitted between animals (*i.e.,* rodents, primates, dogs) by enzootic and epizootic cycles, and humans by endemic and epidemic cycles (Fig. 2B) [18]. Contaminations from animals (*i.e.*, animal-to-animal or animal-to-human) can occur through contact with the fluids of an infected animal, through an animal bite, or the consumption of raw or undercooked meat [35]. The human-to-human transmission has been attributed to the contact with infectious lesions, sore, scabs, or body fluids of an infected individual [27,35]. Recently, sexual intercourse has also been identified as one of the main risk factors [36]. In addition, the infection can occur by other contaminated agents, for example through contact with recently contaminated surfaces or objects. It is important to highlight that poxvirus can remain infectious on surfaces for weeks, depending on the surface and the environmental conditions [37]. However, information regarding the survival time of the monkeypox virus is still scarce.

Monkeypox can be fatal depending on the virus clade, which occurs in 1–11% of cases. For instance, the Central African clade was shown to be more transmissible, with a high mortality rate (approximately 10.6%), while the mortality rate for the West African clade was much lower, affecting approximately 3.6% [38]. Thus, the fatality ratio of the monkeypox virus is varied over the years, ranging in general from 0 to 11% [5]. Currently, no specific treatment for monkeypox infection is reported [30,37]. Nevertheless, the smallpox vaccine can allow cross-protection against another pox-like virus, such as monkeypox. Also, antiviral therapies and vaccines are the greatest weapons against this type of virus infection, which can delay the time to viral emergence by decades or years [20]. Currently, no specific monkeypox vaccines are available, however, it has been observed that minor outbreaks can be controlled by the use of the smallpox vaccine if the vaccine is administered within four days of infection. Thus, smallpox vaccines, such as JYNNEOS™ and ACAM200, have been employed to protect against monkeypox virus infection while the development of new vaccines is studied [39,40]. The fact that smallpox has been eradicated by the World Health Organization vaccination program (Smallpox Eradication Programme) since the 1980s is an excellent example to follow to prevent future Monkeypox epidemics or pandemics.

On the other hand, non-vaccination leads to an increase in human contamination and transmission [19]. We can consider the lessons learned from the recent COVID-19 pandemic, which presented a high rate of contamination among humans and killed more than 15 million

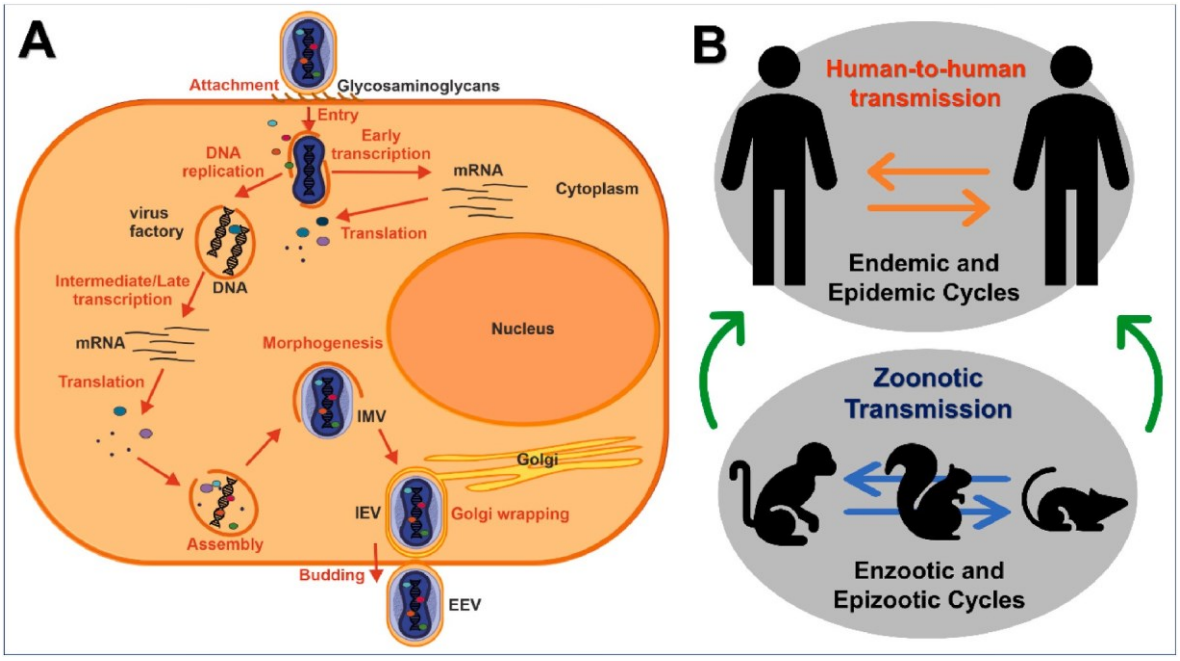

Fig. 2. (A) Representative mechanism of monkeypox virus life cycle and replication after the attachment of a host cell. (B) Monkeypox virus by human-to-human and zoonotic transmission cycles. Reprinted with permission from (A) D. Kmiec and F. Kirchhoff. *International Journal of Molecular Science*, 23(14), 2022. Copyright (2022), MDPI [33].

people worldwide. Monkeypox is considered a neglected and emerging disease, as it has been advancing to other regions and countries. This has raised great concern that it may become the next pandemic.

4. Outbreak of monkeypox

Despite the monkeypox virus being isolated in 1958, the first case of this virus in humans, a nine-month-old boy, was discovered in the Democratic Republic of Congo in August 1970 [1]. Thereafter, there have been approximately 35 outbreaks [41], and the number of cases per 10,000 individuals has been growing considerably with each outbreak, mainly since 1980 [41]. For example, the number of cases increased from 0.72 between 1981 and 1986 to 21.3 and 28.4 per 10, 000 people between 2008 and 2013 [41–43]. However, it is still not clear whether this increase is due to the increase in the contamination rate of the virus by some mutations. Some authors believe this behavior may be linked to the greater number of people moving between different areas of the same country or different countries [44]. In addition, over the years, the concern has been growing. As a result, greater monitoring of the disease reflects the greater number of positive cases detected in each outbreak.

In the USA, an outbreak of human Monkeypox was reported in 2003, the first case of the virus outside Africa (endemic countries) [45,46]. Initially, researchers believed it could be the same type of virus found in different regions of Western and Central Africa after verifying the similarity between the same genomic fragment [46]. However, further studies showed that the virus found in the USA had significant differences in the proteins from the one found in endemic countries [46]. The monitoring of contaminated individuals, as well as a better understanding of the genomic structure of the virus found in the USA and the comparison between other cases of the human Monkeypox virus, allowed the researchers to identify the contamination source [45]. In this case, the transmission was attributed to animal-to-animal and animal-to-human, more precisely after contamination of USA native Prairie dogs, after contact with infected Gambian rats, since they were stored in the same exotic pet trade [18,45]. Those Prairie dogs were later adopted, which ended up infecting 71 humans directly, but no human-to-human infections or deaths from the disease were reported.

More recently, between 2017 and 2018, a new outbreak occurred in endemic countries, such as the Democratic Republic of Congo, the Central African Republic, Cameroon, the Republic of Congo, Liberia, and Nigeria [41]. Although there have already been many other outbreaks in endemic countries, this case is very important because it was so far the largest one, and the first case of human-to-human Monkeypox virus exportation [19,47]. The cases occurred between different states from Nigeria, and different countries, such as the United Kingdom, Israel, and Singapore [19]. During this outbreak, no significant genetic variation was observed between the virus found in Nigeria and the virus in other countries, in the same period, which strongly corroborated the identification of the virus exportation case. On the other hand, the time gap in the occurrence between the outbreaks, and the wide genetic variation in comparison with the virus found in the USA in 2003, suggest that there is no direct link between both, except a common ancestor [19,48].

The latest outbreak of the human Monkeypox virus has been taking place and being monitored by authorities and researchers since May 2022, when in the UK an individual with the monkeypox virus returned from West Africa [33,49]. Since then, more than 9,000 cases have been reported in more than 50 countries [50].

Although the total number of infections has been stabilising worldwide (Fig. 3A), this outbreak has already achieved a total of 75,345 cases, led by the USA, Brazil, and Europe, up to October 20, 2022 (Fig. 3C). However, cases are still growing in some countries, for example in Latin America and Asia [,51]. In the UK, where the greatest number of cases were identified, more than 3,600 people were infected with the human Monkeypox virus, with around 94—96% of the number of cases occurring after sexual relations between men [49,52]. This is a point that has intrigued the scientific community as this outbreak differs from those reported before. In previous outbreaks, a great number of

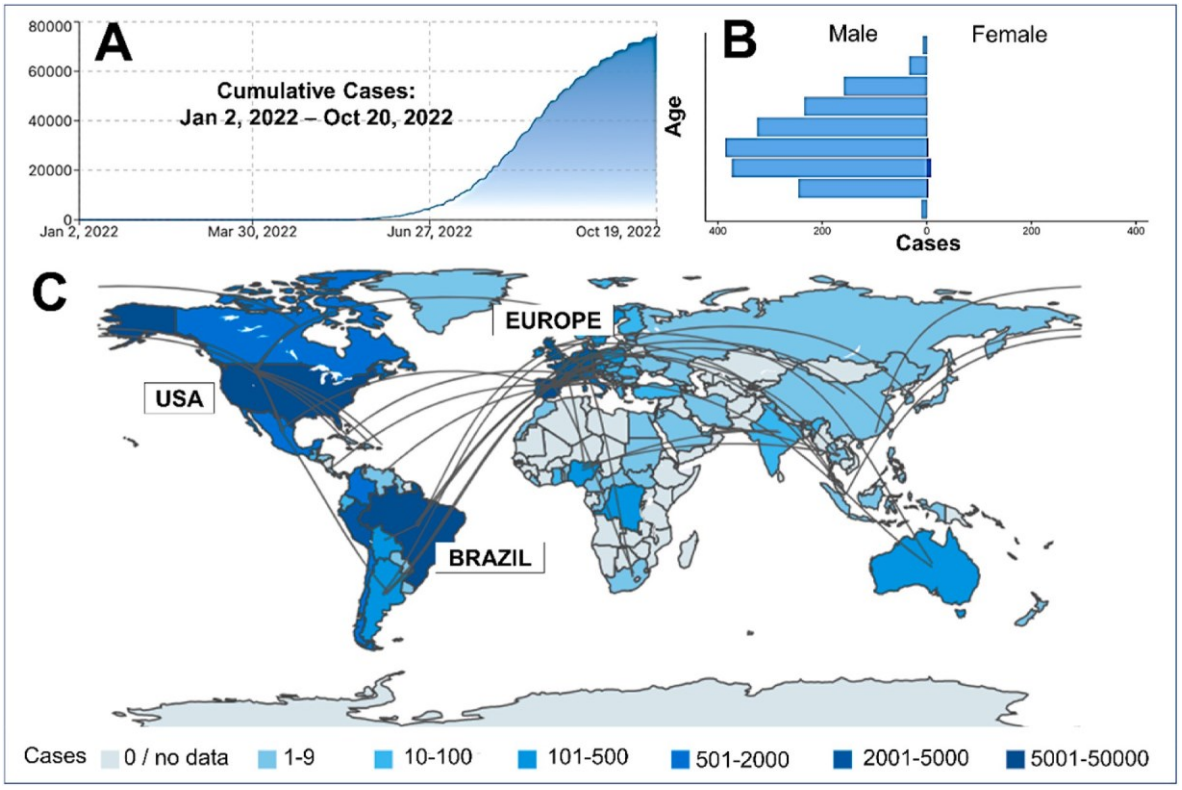

Fig. 3. (A) Total number of human Monkeypox virus cases between January and October 2022. (B) Comparison between the number of cases and gender and (C) cases distribution per country. Adapted from the Global Health GitHub repository, accessed on October 20, 2022 [,53].

infections occurred mainly in young children [49]. In contrast, in this new outbreak, most of the cases have been identified in male adults (Fig. 3B), mainly among homosexuals and bisexuals [49,50,53]. However, the profile of infected people, as well as the main routes of the virus transmission, are still not very clear. Therefore, for this reason, there is a great concern and a need for a better understanding of the disease, such as further investigations about the possibility of transmission by asymptomatic infected people.

Evaluating the profile of human Monkeypox outbreaks over the years, it is evident that the number of cases has been increasing considerably. Numerous factors can contribute to this, such as the increase in population, greater flux of people between endemic and non-endemic regions, and the increase of people monitored at each outbreak, which consequently contributes to increasing the number of positive cases. The Congo Basin (Central Africa) strain and the West African strain of the monkeypox virus are two genetically different strains. Compared to the Congo Basin strain, human infections with the West African strain seem to result in less severe illness [54]. Another important point is the change in the behaviour of the virus in terms of the infection, which was previously only by animal-to-animal and animal-to-human, and mostly in young people and children. Now, the disease is more recurrent in young men and adults after human-to-human contamination. Concerning the contamination after contact with asymptomatic people, further information needs to be investigated, as well as the need for isolation of these individuals, since, in some cases, contaminated people could develop symptoms in up to 21 days [52,55]. However, there is a need for the mass monitoring of the population, which requires a higher production of diagnostic devices with high accuracy, precision, and low cost.

5. Different methods for monkeypox virus detection

For public health and healthcare professionals, monkeypox presents challenges in terms of surveillance and laboratory resources [56,57]. Recent reports indicate that monkeypox, an often-endemic disease, has moved to areas where it is not common due to increased cases in Europe, North America, and Australia. Specific monitoring is made difficult by the fact that its symptoms are similar to those of other viral diseases such as enteroviruses, molluscum contagiosum virus, varicella-zoster virus, and herpes simplex [28,41,56–58]. In addition, many nations do not have the infrastructure to carry out extensive testing [56]. To determine the possibility of infection, tests must be performed based on clinical and epidemiological criteria. Anyone who fits the description of a clinically and epidemiologically suspicious person should be tested. However, testing is not limited only due to resources and equipment, it requires correct means of sample collection and highly trained personnel [56,59,60].

One of the main requirements for performing high-quality tests is a highly qualified and trained workforce [56,61,62]. To carry out the tests, a team with training in handling personal protective equipment is required. In addition, knowing how to correctly handle samples, especially infected materials, and vaccination of health workers is highly recommended, especially for employees who deal directly with infected patients [56,60–62]. Regarding the samples to be collected and handled, the most suitable materials to be used for monkeypox are surface and/or skin materials, such as exudate swabs, and lesion crusts [56,60]. To obtain viral genetic material directly from the lesion, it is necessary for a swab to come into contact with the lesion and to be vigorously swabbed in more than one lesion site. In addition, depending on the case, urine, semen, rectal, and/or genital swab samples may also be collected. However, it is critically important to note that the US Food and Drug Administration is not aware of clinical data to support the use of other types of samples, such as blood or saliva, for monkeypox virus testing [56,57,63]. Test samples not taken from a lesion can lead to false test results. Furthermore, the plasma/serum antibody test alone is not sufficient in the diagnosis of monkeypox, and it is strongly recommended to obtain viral material (DNA) directly from the patient's lesions [56, 61–63].

Considering that the human monkeypox virus has been easily transmitted through contact with body fluids causing public health worries, there is a need to contain the spread of this virus, and the availability of fast and reliable detection methods is of great importance in this scenario. Fig. 4 presents an overview of the main routes for the detection of human monkeypox and the methods to be addressed.

The detection of the monkeypox virus can be performed in conjunction with amplification techniques such as real-time or conventional polymerase chain reaction (PCR), which allows the differentiation between the different poxviruses [64]. Such a test is considered a gold standard test due to its high sensitivity and specificity and has been recommended by the World Health Organization (WHO) for confirmation of monkeypox virus infection [65]. Besides PCR, other types of nucleic acid amplification tests (NAATs) have been explored for the detection of the monkeypox virus, such as Loop-mediated isothermal amplification (LAMP), and Recombinase polymerase amplification assay (RPA) [64,66].

Additionally, strategies including the serological test ELISA (enzyme-linked immunosorbent assay), and even Clustered regularly interspaced short palindromic repeats, in association with the Cas proteins (CRISPR-Cas), have been reported for the detection of this virus [66,67]. In this section, the different techniques for the detection of the human Monkeypox virus detection will be briefly discussed, and perspectives in the development of new point-of-care testing will be covered, focusing on the possibilities to obtain rapid electrochemical devices, as an attractive tool in the diagnosis of monkeypox virus.

5.1. Polymerase chain reaction (PCR)

PCR is a technique to amplify exponentially the sequence of nucleic acids from a target. For this, initially, the DNA is subjected to a cyclic variation of temperatures until it becomes a single-stranded DNA. Then, the annealing of target-specific oligonucleotide primers occurs, providing the amplification by the sequential addition of deoxyribonucleotides which originate a complementary strand, increasing the number of copies [68]. Due to this feature, this technique can increase significantly the sensitivity, allowing the detection of fewer than

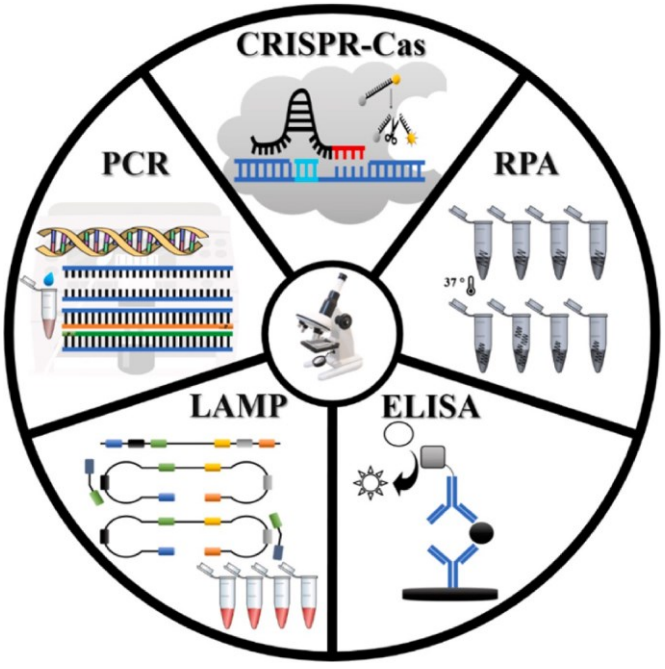

Fig. 4. Main methods used to monitor, detect and control the monkeypox virus.

10 copies of viral nucleic acid [69]. Real-time PCR instruments measure a fluorescence response (accumulation or reduction of fluorescence) of a fluorophore continuously, after each cycle of reaction. The requirement of fluorophores can increase the analysis cost. The analysis of RNA viruses requires the reverse transcription of the genetic material, by the use of reverse transcriptase (RT) enzyme. In this process (RT-PCR), the complementary DNA of the virus (cDNA) is obtained, which is further amplified using the process described previously [68].

Faced with the need for reliable tests that can detect the monkeypox virus, Elbaz et al., 2022, proposed a study that aimed to compare the performance of two commercial real-time PCR assays, the Novaplex™ monkeypox virus assay and the Bio-Speedy® Monkeypox Virus qPCR kit [70]. The main objective was the detection of monkeypox virus DNA from 154 samples and according to the authors, the tests performed demonstrated 100% specificity for the monkeypox virus. The sensitivity values obtained for the assay were 100% and 94% for Novpalex and Bio-speedy®, respectively. Thus, the authors state that the two PCR assays validated provided valuable data that can serve as an important tool for the diagnosis of the monkeypox virus in clinical laboratories.

Though PCR assays are exceptionally sensitive and specific, there are some issues involved in the use of this technique for the contention of viral epidemics. Due to the astonishing sensitivity, the interference by inhibitors of polymerase is a problem, which can lead to false negative results. False positive results can also occur due to possible contaminations; thus, careful handling of the samples, reagents, and product reactions is highly required [68]. Also, the analysis cost is an important factor to be considered. A PCR analysis requires sophisticated installations, instrumentation, and qualified personnel, with this, the analysis becomes costly. In addition, such analysis requires sample collection and transportation, increasing the time for results obtention [71].

5.2. Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification is a nucleic acid amplification technique that replicates a desired target sequence by auto-cycling strand-shifting DNA synthesis by BstDNA polymerase [72]. This approach is classified as simple to apply and effective, requires only simple and cost-effective equipment, can be applied in resource-limited places, and is epidemiologically important for infections caused by the monkeypox virus or other viruses [73]. For this purpose, DNA polymerase is used to carry out amplification under regulated isothermal circumstances with a temperature range of 60 to 65 °. This assay is very specific due to the use of four primers that identify six different target areas (4-primer-based LAMP). Also, by including two "loop primers" in the LAMP test, it is possible to reduce the required reaction time [13,66].

To specifically identify the monkeypox virus, Feng et al. (2022) created a LAMP test. Following that, they assessed the assay's effectiveness using simulated clinical samples and performed naked-eye color detection using fluorescent calcein before each reaction, and monitoring turbidity for comparison [12]. The authors claim that 63 °C was the perfect reaction temperature for the amplification of the sequence and that the primer sets A27L-1 and F3L-1 were the best primers to utilize. As a result, the devised approach had detection limits of 20 copies/reaction mixture for both employing the chosen primer sets. According to the authors, these detection limits were 100 times greater in terms of sensitivity than traditional PCR. Additionally, the LAMP assay was negative for each of the 21 non-monkeypox virus pathogens used in the specificity testing, demonstrating the excellent specificity of the suggested LAMP assay. Furthermore, regarding the simulated clinical samples, the three types were identified by the LAMP assay, and the limits of detection were consistent with the sensitivity results estimated by theoretical sample copies, indicating efficient identification of clinical samples. Finally, the authors described that the fast and reliable monkeypox virus LAMP assay, therefore, can be useful for monkeypox virus detection and on-site diagnosis, especially in primary hospitals and rural areas.

5.3. Recombinase polymerase amplification assay (RPA)

RPA consists of a simple, sensitive, and specific method for DNA amplification. In this method, there is no DNA denaturation and annealing step, and the amplification occurs with the formation of a complex of the primer with the recombinase enzyme. The formed complex begins a strand displacement activity in the double-stranded DNA, providing amplification. After the amplification, a probe, which can be a fluorophore or a quencher, allows real-time detection of the desired fragment. This process can be achieved at a mild temperature range (37–42 °C) within 15 min, which is a great advantage over PCR assays [7,74].

The work of Davi et al. (2019) reports a rapid and specific RPA assay for two clades of the monkeypox virus [7]. According to the authors, the detection occurred within 7 min (while a PCR would take 90 min) with a limit of detection of 16 DNA molecules μL^{-1} in 95% of the cases. Mao and coauthors (2022) developed and validated the use of real-time RPA, and RPA in combination with CRISPR-Cas [14], targeting the gene G2R of the monkeypox virus. The authors reported a limit of detection of 10^0 copies of DNA per reaction, within 20–30 min, with results validated and consistent with the obtained by PCR.

5.4. Enzyme-linked immunosorbent assay (ELISA)

Though orthopoxviruses cannot be distinguished by serological methods, the use of ELISA assays for the detection of the monkeypox virus can be a good alternative for a rapid response [66]. Considering that after the first week of infection, the presence of antibodies is detected, there would be no need for isolation during 21 days, required for PCR testing, and thus, antigen tests are attractive options [66].

ELISA is an immunological assay capable to detect and quantify antigens and antibodies in a sample. This method typically relies on the use of enzyme-labeled antigens or antibodies to quantify the desired species. The antigen-antibody recognition mechanism confers high specificity to the test. When detecting a target antibody, in general, the antigen is immobilized in a microplate, either directly or through a reaction with a specific capture antibody, and the recognition is performed by the sample antibody. A secondary antibody labeled with the enzyme is then added to the test and promotes a color change in the presence of its substrate, allowing the quantification of the desired analyte. [75–77].

Dubois et al., 2011, performed the ELISA assay for specific and sensitive detection of the monkeypox virus [10]. For this, the authors compared two types of peptides, the 20mer and the 30mer. To increase the effectiveness of the proposed assay regarding selectivity and sensitivity, the 30mer peptides were tested together or after the conjugation of selected peptides to a carrier protein (bovine serum albumin). Thus, an optimized combination of four unconjugated 30mer peptides demonstrated a good sensitivity to identify infections that occurred between 2 and 6 months before the test (100%). However, for infections that occurred more than two years ago, the sensitivity value decreased to 45%. Regarding the specificity for the monkeypox virus, the authors stated that the ELISA assay tested provided a value of 99%, demonstrating the high selectivity of the assay, and the high specificity for this assay was only possible due to the use of peptides. On the other hand, an optimized combination of two peptide conjugates provided 90% sensitivity for infections occurring at least 2 years before. Furthermore, even after the new optimization, the specificity level remained high, at 97%. In light of this, the authors stated that peptide-based ELISA tests provide a relatively simple approach to the serological detection of monkeypox virus infection.

5.5. Clustered, regularly interspaced, short palindromic repeat, associated protein Cas (CRISPR-Cas)

Clustered, Regularly Interspaced, Short Palindromic Repeat, also known as CRISPR, is a recent and powerful technology for editing genomes. Due to this ability, applications that include the correction of errors in genomes, treatment, and prevention of infectious diseases, and more recently, clinical diagnosis are possible through this technique [78, 79]. CRISPR-associated protein (Cas) systems (CRISPR–Cas) use RNA-guided nucleases for binding or cleaving nucleic acid sequences [80]. The diagnostic assays performed through CRISPR-Cas are usually performed using Cas12 and Cas13 enzymes [81]. The use of CRISPR-Cas in conjunction with amplification strategies has been performed and is a great alternative to provide an increase in the sensitivity with improved analytical specificity [80].

For the first time, Zao et al., 2022 developed an assay to detect monkeypox virus that combines recombinase-assisted amplification with CRISPR-Cas12a [15]. The monkeypox virus F3L gene was the assay's particular target. Thereby, the CRISPR-Cas12a and amplification approach combination resulted in a low detection limit of 10^1 copies μL^{-1}. The suggested test also demonstrated great selectivity, successfully separating the monkeypox virus from other orthopoxviruses and contemporary high-profile viruses. Finally, the authors created a kit that incorporates lateral flow strips and enables the detection of the monkeypox virus by the naked eye with a LOD of 10^4 copies μL^{-1} to ease on-site screening of possible monkeypox virus carriers. In the same year, 2022, another work reported the use of CRISPR-Cas12a for the detection of the monkeypox virus. Singh et al., 2022, sought to develop an assay based on CRISPR-Cas12a in conjunction with a fluorescent reporter and amplification of recombinase polymerase for the identification and detection of monkeypox virus [16]. To this end, conserved Monkeypox-specific sequences were identified in the polA gene. This gene was chosen because the authors claim that the polA gene differs by a single nucleotide polymorphism from all viruses present in the genus Orthopoxvirus and the use of the polA gene would guarantee a high selectivity of the proposed assay. Therefore, the CRISPR-Cas12a-based assay was able to accurately distinguish the monkeypox virus from other related orthopoxviruses and had a detection limit of 60 copies 1 h^{-1} after the onset of the reaction.

6. Selection of analytes for monkeypox disease diagnosis

The selection of adequate analytes for aiding in the diagnosis of monkeypox disease is vital, assuring that no cross-reactivity will occur with similar viruses or that only the desired strain will be detected. Mainly, the diagnosis of the disease can be performed by two classes of biomarkers: 1) the ones based on the detection of the virus itself or its parts. This includes, for example, viral proteins (A29, I1L, M1L, and others), or specific viral genetic material; and 2) the ones based on the detection of antibodies or other body responses to the infection of the virus. In this case, antibodies are typically specific responses, while other infection biomarkers (for example C-reactive protein (CRP) and interleukins (ILs)) require further investigation for achieving a precise diagnosis. [82–85]. Fig. 5 presents an overview of the main classes of possible analytes for the detection of monkeypox virus. The main specific biomarkers (proteins, genetic material, and antibodies) to be applied for monkeypox diagnosis and their characteristics are discussed below.

6.1. Using monkeypox virus proteins as analytes

Using viral proteins for aiding in the diagnosis of a disease is a strategy commonly applied during infection, usually requiring samples of rash tissue to be performed. As previously mentioned, many monkeypox virus proteins are currently commercially available (IL1, A35, A5L, H3L, C19L, B6R, A33R, M1R, L1R, A29, and more), which might

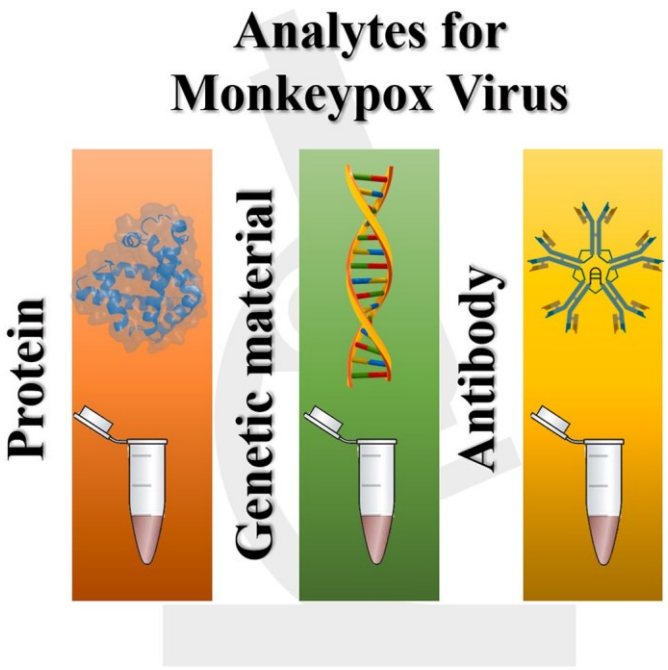

Fig. 5. Main classes of biomarkers for detection of monkeypox virus.

make analyte choice difficult when building a biosensor. It is of interest that a few characteristics are considered when making this choice, including protein concentration, its location on the viral particle, and availability to interact with the receptors. This data, however, is not commonly widely available for many different monkeypox virus proteins, making already validated biomarkers a more practical choice.

The structural similarity with proteins from viruses from the same family is also a very important characteristic to be considered. For example, if the detection of diverse orthopoxvirus is desired or if the analyst is interested in detecting all monkeypox virus strains, a highly conserved protein among the viruses or strains is an interesting choice. In this case, proteins that have an essential function are usually applied. When the detection of a specific strain is desired, however, less-conserved proteins are more interesting. It is important to notice that the use of protein-based biomarkers is more prone to cross-reactivity with similar viruses or strains if compared to the use of genetic material. Therefore, interferences should be carefully analyzed for the desired end.

Some protein-based biomarkers have already been validated for the detection of the virus, including A29, Telomere-binding protein I1 (I1L) and M1R, with commercial ELISA kits being available for these specific targets. One of the most common targets, A29 is a surface envelope protein that binds to heparin and is highly conserved in poxviruses, presenting a 4 amino acid difference from its orthologs [86]. Commercial tests show little to no cross-reactivity between specific anti-A29 antibodies and orthologs [87].

It is important to notice that Wang and coworkers [88] have studied the molecular evolution of monkeypox virus and informed a list of proteins that are more prone to mutations: D2L-like, OPG023, OPG047, OPG071, OPG105, OPG109, A27L-like, OPG153, OPG188, and OPG210 – with those being potential good targets for identifying specific strains. The authors also state that in the monkeypox virus-2022 strains, four and three nucleotide substitutions were observed in OPG105 and OPG210, respectively. It is important to notice that, although such proteins are potential targets for differentiating between monkeypox virus strains (2018 and 2022), these were not commercially found neither the strategy has been validated.

6.2. Using monkeypox virus genetic material as analyte

The approach that holds the greatest potential for high specificity for the detection of viruses is the one based on its genetic material, as biosensors can detect single-base mutations [89]. Furthermore, the amplification of the desired sequence using PCR allows even minimal amounts of viruses to be detected (LOD in the order of fg) [8]. Screening for specific strains is possible and straightforward after mapping the changes in the viral genome, being an interesting tool to understand more about the virus itself, its evolution and its spreading. Identifying the exact virus causing the infection can also be important for medical reasons. For example, the 2022 outbreak virus belongs to monkeypox virus clade 3 (within the formerly designated "West African" clade, which also includes clade 2), with reports showing a <1% case fatality ratio. Clade 1 (formerly designated as "Central African" or "Congo Basin" clade), on the other hand, is considered more virulent and presents a >10% case rate fatality [90].

Using the rationale previously explored, more conserved regions in the genome can be used to detect all monkeypox virus strains or even other viruses from the same family. On the other hand, regions that present higher mutation rates can be used to differentiate between specific strains. For example, a study performed by Li and coworkers [8] after the 2003 monkeypox virus US outbreak showed that a PCR test based on the orthopoxvirus DNA polymerase gene was specific for non-variola Eurasian orthopoxvirus, while a test designed for detecting the monkeypox virus envelope protein (B6R) gene was able to detect 15 different strains of the virus, with no cross-reactivity with other orthopoxvirus or bacteria. Regarding the detection of different monkeypox virus strains, a study developed by Li et al. [91] proposed three different real-time PCR assays. Two of them were specific for the monkeypox virus strain (West African or Congo Basin), while the third one was a generic monkeypox virus assay. Therefore, the careful choice of the DNA sequence to be detected can lead to the development of powerful and extremely specific diagnostic tools for the virus.

6.3. Using anti-monkeypox virus as analytes

PCR- and viral protein-based methods usually require rash tissue to be performed, being restricted to the presence of the virus itself on the body. The detection of antibodies, on the other hand, is an interesting alternative for assessing if an individual was previously sick or vaccinated, and can be especially important to monitor regions that do not possess easy access to diagnostic tests, being a more stable and durable path to assess the viral infection.

Two different types of antibodies develop after infection by monkeypox virus: during the acute phase of infection, the body produces immunoglobulin M (IgM), while later it produces immunoglobulin G (IgG), which remains in the blood even after the infection is cleared. While the presence of IgM indicates an active or recent infection or vaccination, the presence of IgG can indicate infections and vaccinations that happened further in the past. Assessing the change in the levels of IgG can also aid in identifying recent infections [11].

When using antibodies, as when using protein-based biomarkers, cross-reactivity must be considered to assure the construction of an adequate test. As previously mentioned, specific anti-monkeypox virus antibodies are commercially available, for example for protein A29 [87]. Highly specific antibodies are also being constantly researched and developed, allowing the construction of biosensors able to provide more precise diagnostics [92].

7. Rapid point-of-care testing: the use of electrochemical biosensing for monkeypox detection

The need for fast, reliable, accessible, and easy handling and result interpretation platforms proved to be extremely necessary in the past years, with the emergence and reemergence of viral diseases such as human monkeypox. The possibility to perform effective and rapid screening plays an important role in containing the spread of pathogens and even saving lives [93]. The so-called Point-of-Care (POC) devices have been proposed and employed to fulfill this demand. This type of platform consists of diagnostic testing platforms capable to be applied near the patient, outside a centralized laboratory [94,95]. POC tests can contribute to the diagnosis of several medical conditions and can be applied either by specialized staff in medical facilities or by the patient at home, with no need for sample transportation and time-demanding laboratory processing [94].

For a POC device to be considered adequate, some characteristics are targeted. To fill the high demand for pathogens detection and health control, the easy manufacturing process in a scalable way is very attractive. In addition, such devices should be user-friendly and accessible even to resource-limited regions, to reach the greatest part of the population. Another very important feature is the fast and reliable response, allowing an effective screening in a short period [94–96]. The possibility of miniaturization and disposability are also required for the obtention of POC devices. In this aspect, the use of compact instrumentation can reduce costs significantly. In addition, the possibility to use sustainable platforms is also interesting, considering that the disposal of such devices would not harm the environment [97,98].

POC devices can be developed and classified according to the type of signal transduction, which can be electrochemical, colorimetric, fluorescent, or plasmonic [93]. Considering the abovementioned characteristics, one class of sensors stands out to be employed as POC devices: electrochemical sensors. These have several highly attractive and required qualities [99]. Among them, it is worth mentioning the high selectivity and sensitivity, fast responses that can vary from milliseconds to a few minutes, relatively low cost, high capacity for miniaturization and portability, requirements of very low sample volumes, no need for a specialized operator, and being eco-friendly [99–101].

In the literature, there are reports of several POC electrochemical devices for the detection of different types of viruses, such as SARS-CoV-2, Zika, HBV, and Influenza among others [102–111]. Such articles report the assembly of different electrochemical platforms that have high potential to be applied in loco, simply. The use of technologies which include smartphones, microdevices via Bluetooth, microchips, and even portable equipment gathered with a laptop enabled the portability of electrochemical sensors. Fig. 6 presents an illustrative scheme of different types of electrochemical platforms capable of being applied as POC testing for the detection of different types of viruses. As can be seen in Fig. 6, the existing configurations for electrochemical platforms in the detection of viruses are diverse and capable of performing the control of viral diseases effectively and simply. In this aspect, the potential of electrochemical biosensors for detecting the monkeypox virus is highly attractive, since such sensors can successfully meet all the characteristics necessary to carry out the control of this viral disease in areas of difficult access, with a lack of resources and infrastructure, and may be an alternative with great potential, especially in combating inequality in poorer countries.

Although electrochemical biosensors have been widely explored for the detection of viruses, to our knowledge, this tool has been practically non-explored for the detection of the monkeypox virus. However, given the potentialities presented, we believe that electrochemical biosensors could be successfully employed as POC devices for the detection of this virus. In the construction of electrochemical biosensors, different approaches can be explored, depending on the type of viral material to be analyzed [112]. The electrochemical biosensors are analytical devices containing a biorecognition agent bound at an electrically conductive surface, which reacts selectively with the analyte of interest. The biorecognition event generates electrical responses which are sensitive to the concentration of the analyte, enabling its quantification [112]. The use of genetic material (DNA/RNA) can be explored in the construction of genosensors and aptasensors. In the case of aptamer-based biosensors, single-stranded artificial nucleotides (DNA or RNA) capable of

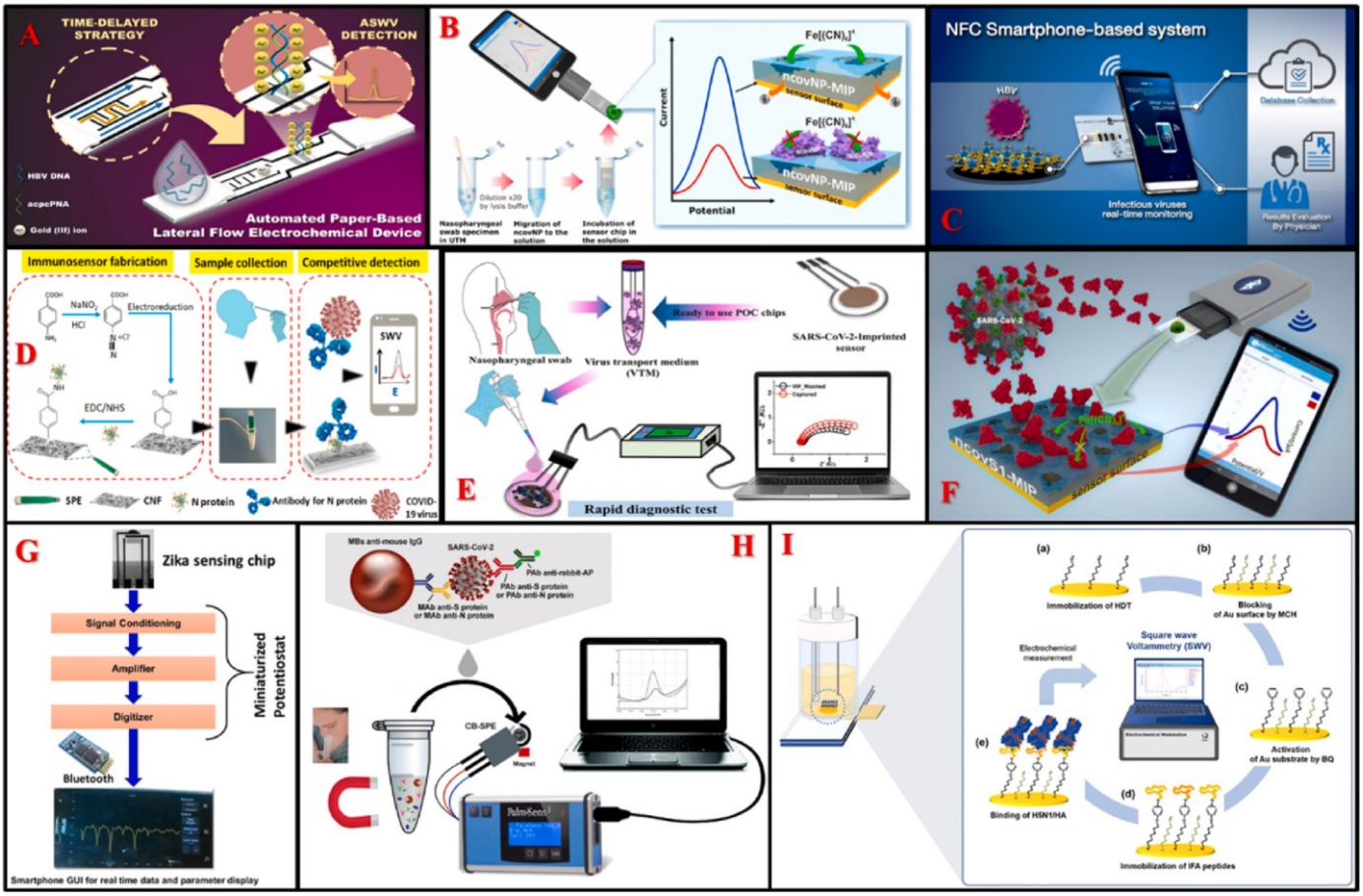

Fig. 6. (A) Schematic Illustration of the eLFA Device for One-Step HBV DNA Detection. (B) COVID-19 diagnostics principle by nconNP sensor analyzing the samples prepared from nasopharyngeal swab specimens of patients. (C) Illustrative scheme of NFC smartphone-based system portable potentiostat. (D) Schematic of the Cotton-Tipped Electrochemical Immunosensor for COVID-19; sample collection functionalization of the biosensor and detection principle. (E) Steps of SARS-CoV-2 VIP Sensor fabrication to create a Template for the specific recognition of the SARS-CoV-2. (F) Illustrative scheme of the smartphone-based electrochemical platform and the operating principle of ncovS1 sensor in COVID-19 diagnosis. (G) Roadmap to develop an electrochemical ZIKV immunosensor at POC application. (H) The MBs-based assay for SARS-CoV-2 detection in untreated saliva. (I) Schematic illustration of the electrochemical sensor detecting the influenza virus HA antigen. Reprinted with permission from (A) Srisomwat, C. et al. Anal. Chem., 93, 2879, 2021. Copyright (2020), American Chemical Society [102]. (B) Raziq, A. et al. Biosens. Bioelectron, 178, 113029, 2021. Copyright (2021), Elsevier [103]. (C) Teengam, P. et al. Sensors Actuators B Chem., 326, 128825, 2021. Copyright (2020), Elsevier [104]. (D) Eissa, S. et al. Anal. Chem. 93, 1826, 2020. Copyright (2020), American Chemical Society [105]. (E) Hussein, H.A. et al. ACS Sensors, 6, 4098, 2021. Copyright (2021), American Chemical Society [106]. (F) Ayankojo, A.G. et al. Sensors Actuators B Chem. 353, 131160, 2022. Copyright (2021), Elsevier [107].
(G) Kaushik, A. et al. Sci. Rep., 8, 9700, 2018. Copyright (2018), Nature [108]. (H) Fabiani, L. et al. Biosens. Bioelectron, 171, 112686, 2021. Copyright (2020), Elsevier [109]. (I) Kim, J.H. et al. Sensors Actuators B Chem. 343, 130161, 2021. Copyright (2021), Elsevier [110].

specifically binding to a target are employed [99,113].

Genosensors are similar to aptasensors, and consist of devices that combine a biological recognition agent (typically ssDNA) that confers high selectivity for the desired target and a transducer that provides the necessary sensitivity and converts the detection event into a measurable electronic signal, which we can relate to the presence of the desired target and its concentration [99]. In general, one of the main characteristics of genosensors is their simplicity, which generally requires a few steps to measure the target of interest. In addition, electrochemical genosensors have high sensitivity, extremely low LODs, and generally do not require complex pre-treatment of the sample [99,101]. However, in some cases, this type of sensor has some disadvantages, mainly with greater instrumental complexity, as they often need to be used in conjunction with DNA amplification techniques [99]. In addition to these two classes, there are also electrochemical immunosensors. Immunological tests can also be performed using electrochemical immunosensors, and they eliminate the need for amplification methods. In this type of biosensor, the biorecognition element consists of antibodies or antigens, and the immunochemical reaction provides a disturbance in the electrical response (increase or decrease), proportional to the analyte concentration [112,114]. Fig. 7 shows a schematic representation of biosensors involving electrochemical transduction.

The literature reports the use of electrochemical genosensors for the detection of several viruses. Among the various architectures for the construction of genosensors, simple approaches using low cost and portable materials have drawn the attention of the scientific community. In light of this, a highly attractive paper-based genosensor was presented by Srisomwat et al., 2020 for hepatitis B virus detection [115]. The authors developed a pop-up DNA device that can serve as an alternative 3D microfluidic paper-based bioanalytical device (μPAD) for the detection of unmarked Hepatitis B virus (HBV) DNA. Fig. 8 presents a schematic representation of the pop-up PAD DNA platform in "open" and "closed" formats and a summary illustration of the genosensor fabrication steps, as well as the analytical responses obtained at each step.

The main objective of the pop-up design is to allow the control of the fluid path, incubation time and electrical connectivity simply and practically. The platform proposed by the authors allows for performing the analysis in several stages in a single device. In addition, this

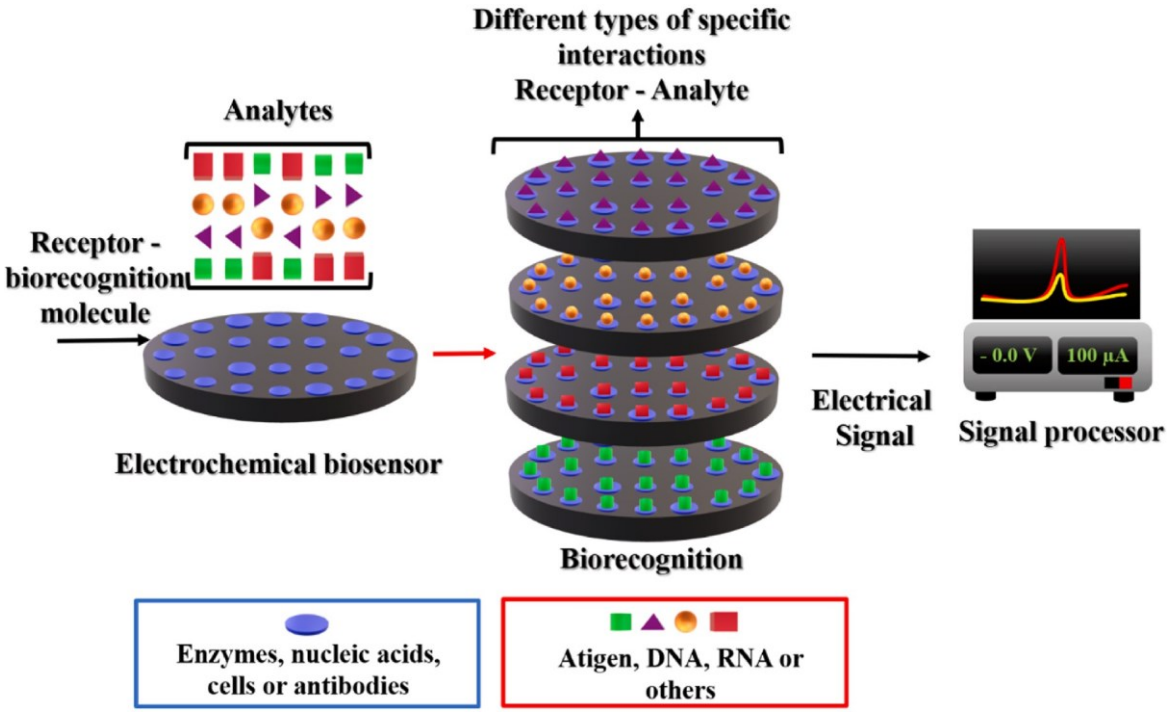

Fig. 7. Scheme representative of a biosensor with the electrochemical transducer.

architecture allows for easy sample introduction, avoiding contamination and minimizing biofluid exposure. The genosensor was based on the analytical method of detection called signal-off, which is widely used in the construction of electrochemical biosensors. The method is based on the fact that as the modifying layers are added (biorecognition agents or not) the analytical signal from the sensor in the presence of a redox probe will change. In the stage of biorecognition of the analyte of interest (in this case, the HBV virus DNA), the analytical signal in the presence of the redox probe will suffer a current decay, which can be measured and correlated with the concentration of the analyte of interest (HBV DNA). The electrochemical sensor modification strategy is very simple. Initially, the immobilization of Pyrrodinyl peptide nucleic acid (acpcPNA) on the surface of the graphene ink sensor by covalent bonding was carried out. Pyrrodinyl peptide nucleic acid has specificity for hybridization with HBV DNA, thus, in the presence of HBV DNA, the hybridization with the Pyrrodinyl peptide nucleic acid present on the surface occurs, partially blocking the surface of the electrode and causes a current decay that can be related to the concentration of HBV DNA. Therefore, the authors stated the construction of a successful electrochemical genosensor, capable of detecting the DNA of the HBV sensitively and selectively. In addition, the developed device has the potential to perform POC analysis.

Additive manufacturing (3D printing) technology is also present in the construction of electrochemical genosensors. Due to the qualities attributed to additive manufacturing such as large-scale manufacturing capacity, reduced cost, and the possibility of producing miniaturized sensors with different designs [116], this technology is a strong ally in the development of genosensors. Silva et al., 2022 presented the development of a genosensor manufactured from 3D printing with conductive graphene filament for the detection of complementary DNA (cDNA) targets of the SARS-CoV-2 virus [117]. The detection method was also based on signal-off response. The fabrication of the genosensor was based on anchoring a capture cDNA strand on the surface of an electrochemically gold-modified 3D printed sensor. Fig. 9 shows the 3D-printed genosensor fabrication steps. The anchoring of the capture cDNA is possible because the single strands of cDNA have a thiol group, thus being able to bind to the gold on the surface of the electrode simply and easily. Finally, the captured cDNA present on the surface of the sensor hybridizes with the target cDNA from the SARS-CoV-2 virus. Therefore, due to the partial blocks caused by the anchoring of the capture cDNA and hybridization with the capture cDNA of the SARS-CoV-2 virus, the analytical signal of the sensor decays in the presence of a redox probe. In this way, it is possible to detect and quantify the target cDNA of SARS-CoV-2 present in the samples.

As can be seen, electrochemical genosensors are simple to manufacture, which is why their potential is enormous. Methods of producing genosensors only by anchoring the single-stranded (ssDNA, as known as probe) on the surface of the sensor are widely used. In addition, the anchored ssDNA used will give the genosensor high specificity, which is required in clinical tests [118]. To anchor the probe on the electrode surface, different approaches can be employed, which include adsorption, crosslinking, complexation reactions, or covalent attachment [119]. The adsorption method is the simplest route, though desorption can occur depending on the hybridization conditions, in addition to the non-specific bonding of the DNA target to the electrode due to strong interaction (chemisorption). An interesting option to provide the probe attachment would be through the use of thiolated DNA, which can be obtained through the use of mercaptoalkyl linkers [118]. In this case, thiol groups are added to the DNA terminations and, with this, covalent bonding can occur with gold surfaces or composed of other metals, such as silver and platinum [120,121]. Thus, the use of gold or gold-modified surfaces can be efficiently used for probe attachment at the electrode surface [118,122]. Surface modification with gold particles or nanoparticles is highly widespread, well-established in the literature, and simple to perform [123]. After immobilization, the hybridization process can provide specific bonding, and the transduction can be easily performed. In this sense, the signal-off detection strategy, mentioned previously, is highly effective and simple. Therefore, the production of a genosensor for monkeypox virus detection could be performed through the use of a thiolated probe, using a gold-modified surface, following the protocol described. Finally, given the practical proposed manufacturing process and interpretation of results, the genosensors can be applied as

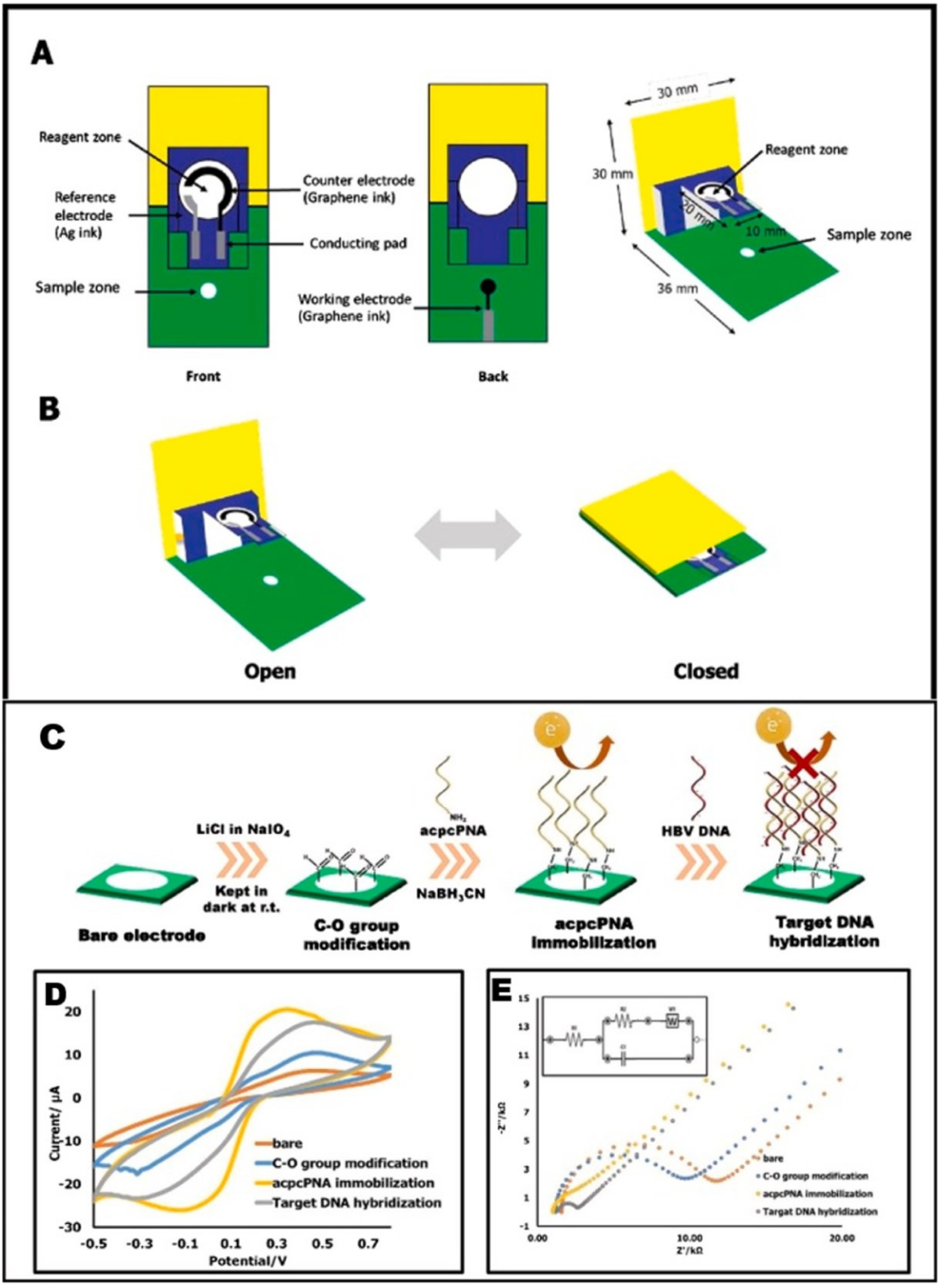

Fig. 8. (A) Representation of the pop-up PAD DNA sensor; (B) Pop-up PAD DNA sensor in the "open" and "closed" configurations; (C) Representative scheme of the DNA genosensor fabrication; (D) Cyclic voltammograms and (E) Nyquist diagrams for the bare electrode, electrode in the presence of the aldehyde group modification, and the acpcPNA immobilization before and after hybridization with one equivalent of HBV DNA in presence of $[Fe(CN)_6]^{3-/4-}$. Adapted with permission from Srisomwat, C. et al. Sensors Actuators B Chem. 316, 128077, 2020. Copyright (2020), Elsevier [115].

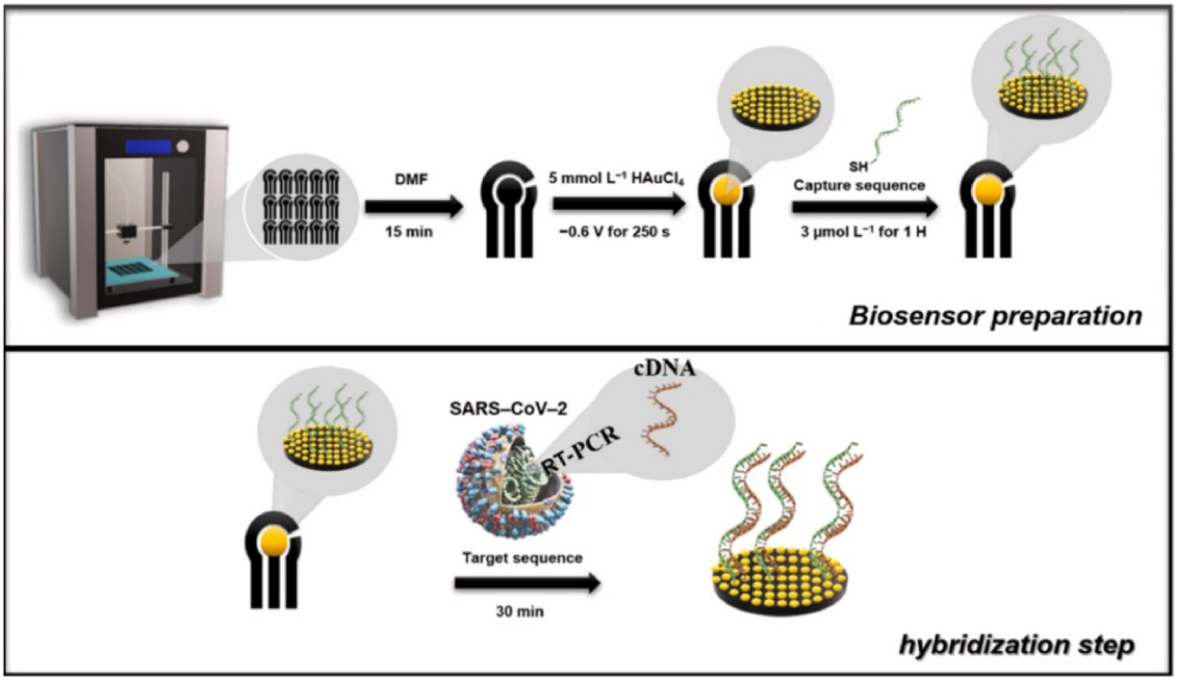

Fig. 9. Schematic representation of the genosensor production and DNA hybridization step. Reprinted with permission from Silva, L.R.G. et al. Biosensors, 12, 1, 2022. Copyright (2022), MDPI [117].

POC devices.

Aptasensors have also been reported in the literature for the detection of different viruses. In the work of Bachour Junior et al., 2021, an electrochemical aptasensor to detect the dengue virus through the NS1 serotype 1 and 4 proteins in undiluted human serum in the clinical range was developed [124]. The choice of the NS1 protein is due to its high levels in human blood after contamination with the dengue virus in the initial phase. The biosensor was based on gold electrodes co-immobilized with specific aptamers and 6-mercapto-1-hexanol (MCH) to obtain a self-assembled monolayer. In addition, the method of obtaining the analytical signal was also based on the signal-off, as it is a highly efficient and simple method to be interpreted. Fig. 10 presents a schematic illustration of aptasensor preparation. As shown in Fig. 10, a mixture containing MCH and the aptamer with specificity for the NS1 protein was initially prepared. It is important to emphasize that both compounds have thiol groups at the terminations. The MCH serves as a spacer and self-assembles the aptamers on the surface of the gold sensor, giving them an organized arrangement. In addition, Bovine Serum Albumin (BSA) protein is used to block non-specific sites so that parallel reactions do not occur.

After the assembly of the electrochemical aptasensor, the aptamers bind to the NS1 protein and, based on the increase in resistance (due to the partial blocking of the surface), the authors were able to detect and quantify NS1 protein in human serum. Furthermore, according to the authors, NS1 levels in human serum are ranging between 0.04 and 2 µg mL^{-1} in the first and 0.01–2 µg mL^{-1} in the second infection. The authors point out that the developed sensor, can detect these concentrations in undiluted human blood and, therefore, may be a highly viable alternative for the clinical detection of the dengue virus. Furthermore, aptasensors also have great potential for possibly detecting the

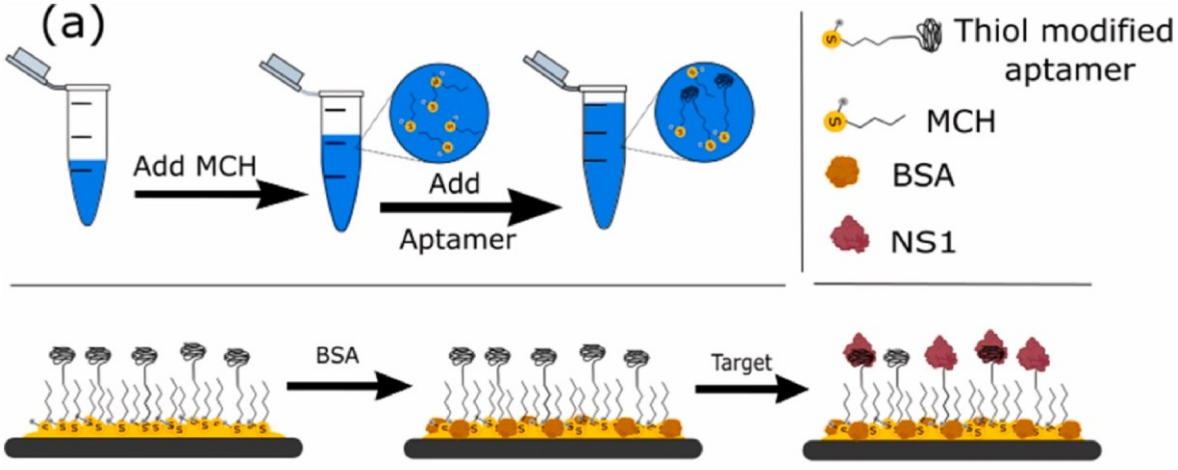

Fig. 10. (a) Illustration of co-immobilization solution preparation and surface architecture on top of the gold electrode after BSA blocking and NS1 binding. Adapted with permission from Bachour Junior, B. L.R.G. et al. Talanta, 233, 122527, 2021. Copyright (2021), Elsevier [124]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

monkeypox virus, as they are easy to assemble and have a simple analytical response. Thus, the production of aptasensors for specific analytes that indicate contamination of the individual is possible, whether these analytes are proteins or another specific biomarker. It is important to notice, however, that no specific aptamer for monkeypox virus was yet developed, to the best of our knowledge.

Immunosensors for the detection of the monkeypox virus can also be a great option as detection platforms. Literature shows interesting works reporting the easy obtention of immunosensor for different types of viruses. Mojsoska et al., 2021, proposed a label-free electrochemical immunoassay for the rapid detection of the spike protein of the SARS-CoV-2 virus. For this, the authors used a carbon screen printed electrode (SPE) functionalized with a monoclonal anti-spike antibody. The graphene electrode surface was functionalized with 1-pyrene butyric acid N-hydroxysuccinimide ester (PBASE), bound to the antibody. After immobilizing the antibody, an incubation time of 45 min was sufficient for the immunoreaction with the spike protein antigen, and the electrochemical response can be obtained, linearly responding to the concentration of antigen incubated. Fig. 11 shows the steps involved in the construction of the immunosensor. The electrochemical response obtained in this case is a perturbation in the current response of a redox probe, in this case, $[Fe(CN)_6]^{3-/4-}$. The immunosensor provided specific responses, in the concentration of 260 nmol L^{-1} (20 μg mL^{-1}), and the time response was significantly lower than the required for PCR analysis, employing a portable device that can be employed on-site.

In another work, Beduk et al. also performed using an electrochemical immunosensor for SARS-CoV-2 S-protein detection [126]. The authors used a Laser-scribed Graphene (LSG) electrode as a biosensing platform. The LSG-based electrode was obtained from a Polyimide (PI) substrate and the laser patterning was obtained with a CO_2 laser. The electrode surface was further modified with gold nanostructures and with cysteamine and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide: N-hydroxy succinimide (EDC:NHS) as cross-linking agents, responsible to create electroactive groups and consequently allowing the immobilization of the biomolecule. The antibody was directly immobilized at the EDC:NHS, and a BSA blocking was then performed, providing an immunosensor ready to use. The incubation of the antigen occurred within 1 h, thus, the analysis is considerably faster than PCR. The system was integrated into a smartphone, providing an integrated POC system for the diagnosis of the SARS-CoV-2 virus. A detection limit of 2.9 ng mL^{-1} was achieved. Fig. 12 summarizes the biosensor assembling process and analysis step.

As can be seen, the obtention of electrochemical immunosensors is considerably simple. The use of such biosensors for the detection of the monkeypox virus may be affordable and attractive. Similarly, to the presented case of genosensors, a protocol for the obtention of monkeypox virus electrochemical immunosensor can also be established. In this case, it would be necessary the use an electrode base, which could be a carbon-based electrode. The use of carbon electrodes is very common, due to the astonishing properties of such materials, such as good electrical conductivity, inertness, low cost in some cases, and the possibility of functionalization. The use of EDC:NHS, as explored in the work of Beduk et al. [126] can be easily used to allow covalent bonds with the amines present in the antibodies, thus, the immobilization of EDC:NHS

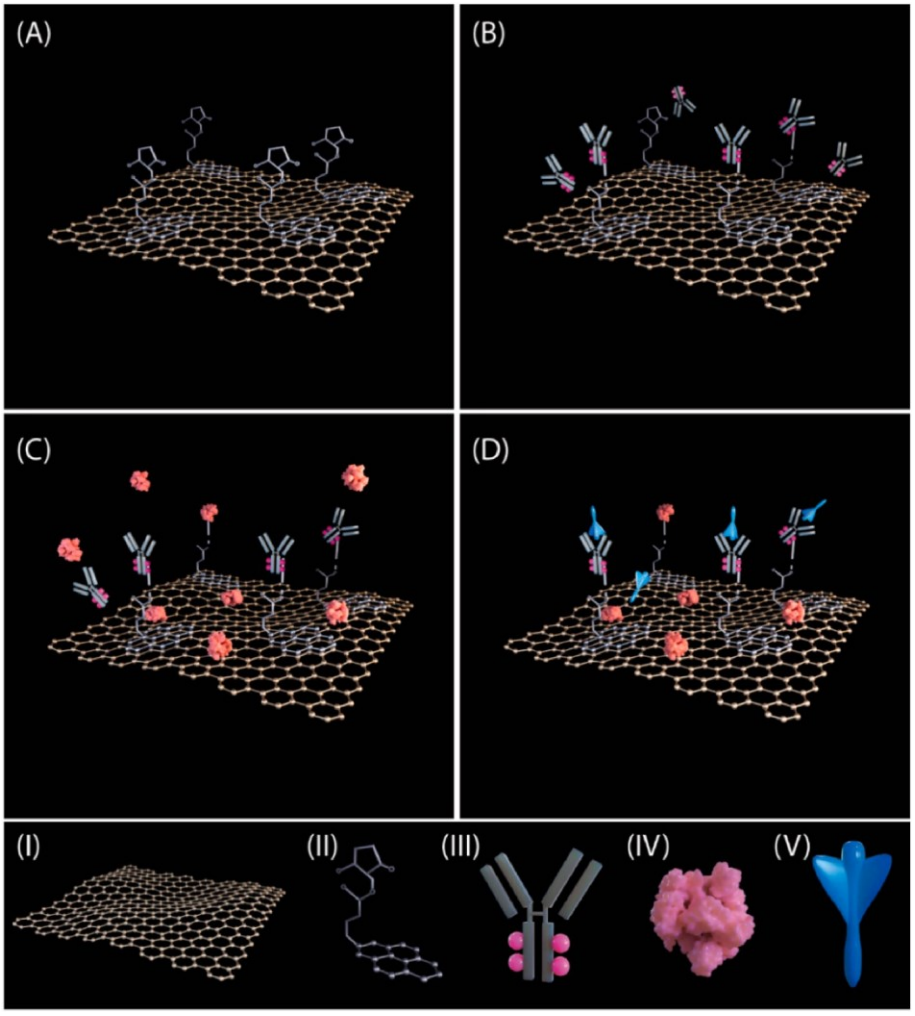

Fig. 11. Schematic representation of the obtention of an electrochemical immunosensor for SARS-CoV-2 virus detection; (A) Functionalization step; (B) Immobilization of spike-specific antibodies to the electrode using the linkers; (C) Blockage of the electrode using BSA; (D) Incubation of the spike protein upon the biosensor. (I) Graphene lattice; (II) cross-linker; (III) spike-specific antibody; (IV) BSA protein; (V) SARS-CoV-2 spike subunit 1 protein. Reprinted with permission from Mojsoska, B. et al. Sensors, 21, 390, 2021. Copyright (2021), MDPI [125].

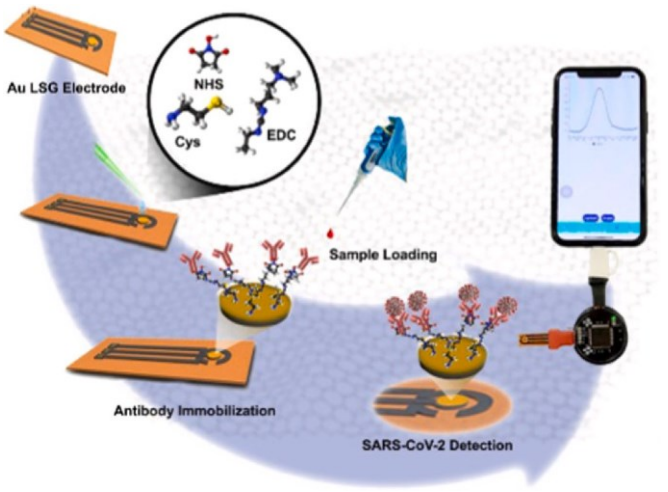

Fig. 12. Schematic representation of the obtention of an electrochemical immunosensor for SARS-CoV-2 virus detection using LSG electrode. Reprinted with permission from Beduk, T. et al. Anal. Chem, 93, 858521, 2021. Copyright (2021), American Chemical Society [126].

at the electrode surface is a way for obtaining the electrochemical immunosensor. To immobilize EDC:NHS, the presence of carboxylic groups at the electrode surface is required, and these functional groups can be obtained after the functionalization of carbon-based electrodes [127]. Considering that different types of monkeypox virus proteins are commercially available (A35, A29, H3L, L1R, A33R, B6R, among others), specific antibodies for the recognition of these proteins can be acquired commercially. With this, an electrochemical immunosensor for monkeypox virus detection can be obtained.

As can be seen, electrochemical biosensors are promising tools to be applied for monkeypox virus detection. Different biomaterials can be employed in the construction of such platforms, attributing selectivity to the analysis. High sensitivity, fast responses miniaturization, and portability make electrochemical platforms ideal for the construction of POC devices. Another astonishing feature related is the versatility allowed by the obtention of electrochemical biosensors. The possibility to employ a great variety of materials can attribute versatility in costs, in addition to characteristics such as flexibility (ideal for the construction of wearable sensors), resistance, and durability. In addition, there is the possibility to obtain platforms with varied structures and sizes that can be molded according to the desired (e.g. when using 3D printing technology in the construction of platforms).

In the context of the potential of state-of-the-art electrochemical biosensors for monkeypox determination, it should not be overlooked the role and versatility of use that the latest generation of nanostructured and/or multifunctional bionanomaterials are demonstrating in empowering the attributes of these biodevices in terms of sensitivity, selectivity, simplicity, response time, and antifouling ability, making them suitable for getting a foothold in real-world applications [128].

Among these modern biomaterials, which are demonstrating competitive advantages over other commonly used bioreceptors or artificial nanomaterials, arousing the scientific community's enthusiasm for their wider exploitation, polyA-type probes, nanostructured and multifunctional or multimeric peptides and aptamers, used as recognition elements, electrode modifiers (acting as linkers or creating scaffolds with antifouling properties), enzyme substrates (in the case of peptides), and labeling/carrier agents for signal amplification, deserve to be highlighted [99,129].

Although so far in connection with electrochemical sensing, only multifunctional aptamers and peptides have been incurred the detection of viruses, and in particular of spike proteins of the wildtype SARS-CoV-2 virus and Alpha and Delta variants [130] and COVID-19 N-gene [131], respectively, polyA probes, both single [132] or multiblock [133] type, exploited in the determination of bacterial genetic material would be perfectly also transferable to the determination of viral genetic material. It is also readily deducible from recent literature that modern peptides and multifunctional aptamers [134,135] would also impart unique features to electrochemical biodevices for the determination of viral antigens and the antibodies that our immune system generates to protect us against them.

8. Conclusion and prospects

The early and fast detection of infectious diseases is crucial. The availability of highly versatile techniques, with the potential to be readily adapted to provide new diagnostics platforms for viral diseases, is outstanding. Concomitantly with a global pandemic caused by the SARS-CoV-2 virus, the reemergence of the human monkeypox virus increased considerably the worries about public health. Therefore, bringing light to new methods for detecting the monkeypox virus is essential for public health, especially methods that can be applied as POC devices. In addition, such methods need to be applied in countries with less infrastructure, especially in poor communities, so they can build a fairer and more inclusive health policy.

Traditional methods for viral disease detection, such as PCR and ELISA play an important role in the containment of viral diseases. Their astonishing sensitivity and robustness are essential. However, the search for faster and decentralized diagnostics is also required to serve as trial tools or complete detection methods. In this aspect, electrochemical biosensors stand out. In the construction of electrochemical sensors, a great variety of materials can be employed, and due to this, flexibility, resistance, electrical conductivity, miniaturization, and even low cost can be easily attributed to the final platform. In addition, there is freedom in the obtention of the final design, which can allow the obtention of POC platforms or even wearable devices, according to the need. Following easy protocols, we believe that it is possible to obtain electrochemical biosensors for the detection of the monkeypox virus.

In addition, it is important to highlight the use of biosensing-adapted electrochemical platforms that can reduce the sample volume required and minimize the contact of the operator with infected samples. In this aspect, 3D-printed sensors present an ideal capability. Besides 3D printed sensors, disposable sensors such as those produced based on conductive inks, laser scribing, and pencils drawn also highlight extremely positive points, as they can be discarded after a single use since they are made of recyclable materials and are very low cost. However, regarding an ideal scenario, the manufacture of electrochemical tests for the monkeypox virus goes beyond electrochemical sensors, the production of miniaturized signal decoding equipment that can be used coupled to a smartphone or remotely is also a key point for the success of the electrochemical biosensing platforms. In this context, the utilization of different, easily accessible materials and technologies such as smartphones, enables the development of electrochemical biosensors for monkeypox virus, particularly in countries with limited infrastructure, potentially facilitating extensive production of tests for the monkeypox virus, thereby contributing to public health and equity. Thus, by joining high-quality electrochemical sensors with desirable POC characteristics, with miniaturized equipment for on-site application, the potential of the tests will be extremely high, guaranteeing the possibility of sensitive, selective, fast, and simple analysis.

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.

Data availability

Data will be made available on request.

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