Addressing the Silent Spread of Monkeypox Disease with Advanced Analytical Tools

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Nikhil Bhalla and Amir Farokh Payam**

Monkeypox disease is caused by a virus which belongs to the *orthopoxvirus* genus of the *poxviridae* family. This disease has recently spread out to several non-endemic countries. While some cases have been linked to travel from endemic regions, more recent infections are thought to have spread in the community without any travel links, raising the risks of a wider outbreak. This state of public health represents a highly unusual event which requires urgent surveillance. In this context, the opportunities and technological challenges of current bio/chemical sensors, nanomaterials, nanomaterial characterization instruments, and artificially intelligent biosystems collectively called "advanced analytical tools" are reviewed here, which will allow early detection, characterization, and inhibition of the monkeypox virus (MPXV) in the community and limit its expansion from endemic to pandemic. A summary of background information is also provided from biological and epidemiological perspective of monkeypox to support the scientific case for its holistic management using advanced analytical tools.

1. Introduction

Human monkeypox is a disease caused by virus which belongs to the genus o*rthopoxvirus*, that also includes camelpox, cowpox, vaccinia, and variola viruses as other diseases.[1] The disease was first discovered in 1958 with and, to some extent, non-human primate species in African continent.[2] However, until 1970 monkeypox was not recognized as a distinct infection in humans. Presumably, there were infections before 1970, but these infections were probably concealed by smallpox which was widely endemic at that time.[3] However, since 1970s the infections in humans have escalated with reports of human to human transmission. Apart from smallpox eradication, increase in monkeypox infections can be attributed to a number of factors such as advancement in detection technologies, surveillance efforts, climate change (that leads to environment degradation), and urbanization of human settlements where this virus is maintained in its animal reservoir(s) and, consequently, serve as a source for human infection. Geographically, the human infections were mostly restricted to specific regions of Africa until very recent.[4] However, since the start of 2022, infected cases of monkeypox virus (MPXV) have appeared intercontinentally, especially in Europe and North America, suggesting that the spread of human monkeypox infections may continue to rise in near future.[5] Currently, as on 25 October 2022, there are more than 75 000 cases reported in the 2022 MPXV outbreak. These cases are distributed in 109 countries among which only seven countries have reported MPXV before 2022. Among these cases, 34 deaths have been reported so far this year in 12 countries, see more details in **Figure 1**.

This spread can be due to the genetic predisposition of the MPXV which enable it to infect several animal species in different geographic regions. Additionally, the use of vaccines VACV, vacccina virus and MVA-BN, modified vaccina ankara-bavarian nordic), widely used for smallpox, and one drug treatment using tecovirimat have been confirmed for monkeypox, in 2019 and 2022, respectively. However, these counter steps are not yet available widely.[6] Moreover, vaccination is becoming a less feasible option to control viral infections in today's fast increasing immuno-compromised population in particular with the emergence of coronavirus 2019 (COVID-19).[7] An increased occurrence of human MPXV infections in immuno-compromised population, may permit MPXV to mutate and maintain itself independently in community.[8] This suggests that there are several unmet challenges which are currently present for the scientific community to timely limit and control the monkeypox disease spread, before it becomes pandemic. While the current risk associated with MPXV spread is considered as "moderate" by the world health organization (WHO), the fact that it is emerging in a diverse geographical location suggest that the virus has gone undetected in the past. Within this context, we review recent advances in advanced analytical

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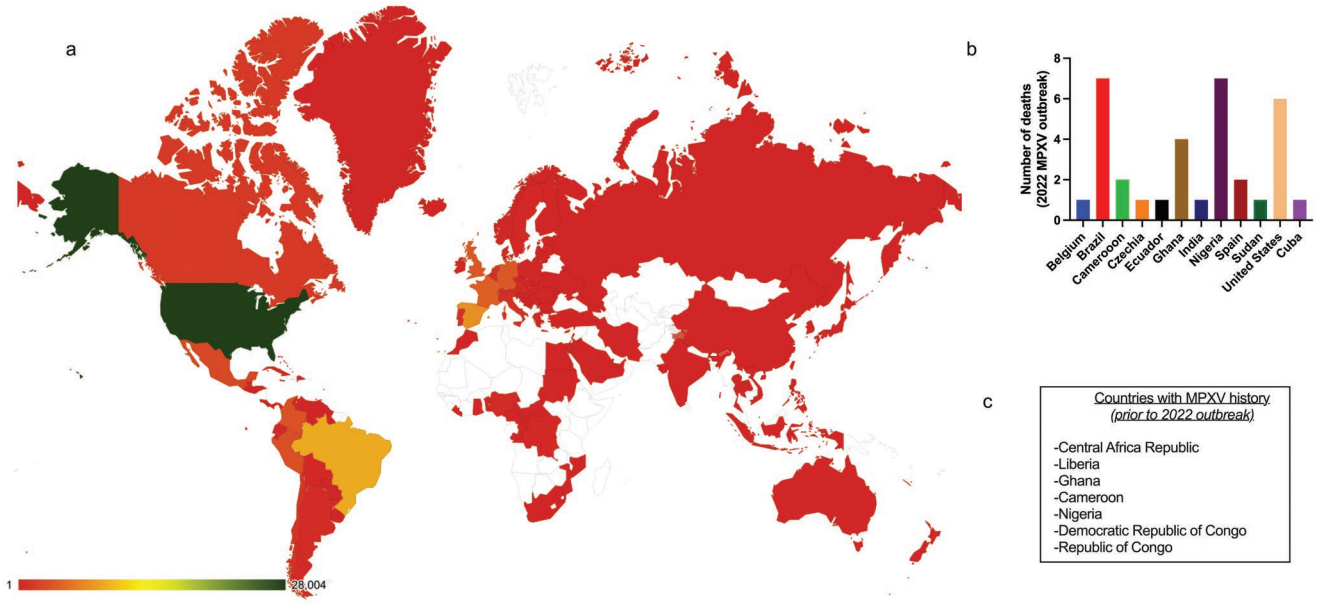

Figure 1. 2022 MPXV outbreak statistics a) shows distribution of infected cases across the globe; b) shows number of deaths due to MPXV reported before 25 October 2022; and c) depicts countries where MPXV was reported prior to 2022 outbreak. Source: WHO, European CDC, US CDC, and Ministries of Health; link to source https://www.cdc.gov/poxvirus/monkeypox/response/2022/world-map.html.

tools (collective name for nanomaterials, nanomaterials characterization instruments, and artificially intelligent biosystems referred in this work) which can be used for early detection and management of the monkeypox disease. The review also highlights potential lessons (from the perspective of sensor development and quick characterization) which can be adopted from COVID-19 management using technology to limit the spread of monkeypox disease.

2. Features of Monkey Virus and Current Detection Technologies

Prior to the discussion on nanotechnology solutions for tackling MPXV we share various structural, physical, and chemical characteristics of the MPXV and symptoms in patients which facilitate its detection. This is followed by discussion on the existing detection technologies for the virus and their limitations which allow room for development of advanced but low-cost nanotechnologies.

2.1. Disease Etiology and Current Diagnosis

In the initial stages of the disease, it causes symptoms which are like influenza such as headache, fever, and chills. Within days patients develop distinctive rashes on the body which starts to form painful lesions.[9] These painful lesions are the hallmark of the disease and can cause scaring. Though the virus strain causing this disease leads to mild symptoms in the patients, it has also been reported of fatal consequences. The virus has been reported to spread by direct contact, contaminated objects, and through respiratory droplets.[10] Most of these symptoms (also common to other viral/bacterial diseases) are usually detected by the infected individual themselves using a thermometer or by observation of changes in the physical/metabolic state of the body. Upon identification of these symptoms, clinical tests are conducted on the suspected patients which confirm the presence/absence of the monkeypox disease.

Currently, it is the polymerase chain reaction (PCR), which is the most preferred laboratory test for MPXV, given its accuracy and sensitivity detection of viral genetic material.[11] To conduct this test, fluid from skin lesions or the dry crust of the lesion (biopsy where feasible) itself are taken from the patients for analysis. In some cases urine or other body fluids are also reported to be used for PCR analysis, see **Table 1** for more details. Note that PCR blood tests are less preferred for MPXV detection due to short duration of viremia relative to the timing of specimen collection after symptoms begin to appear.[12]

After collection of the sample, the assessment of the monkeypox infection is justified by nucleic acid amplification testing (NAAT). This uses real-time or conventional PCR, for detecting the specific sequences of current MPXV DNA. The complete genome sequence of one of the MPXV strain in current outbreak (isolate name MPXV_USA_2022_MA001) is available in the GeneBank, which is a public database of DNA sequences hosted by the National Center for Biotechnology Information (NCBI) at the National Library of Medicine (NLM), USA (Accession number: ON563414). When there is no availability of a MPXV specific PCR test, a positive PCR for orthopoxvirus can be considered for confirmation of MPXV virus in non-endemic countries, as there is very less/no circulation of other orthopoxviruses in community. The antibody detection from blood which is a common bioassay for many diseases is also less preferred as a standalone diagnosis method for monkeypox detection. This is attributed to the fact that smallpox vaccination,

Table 1. Specimen collection and handling.

Specimen type	Minimum volume	Collection method	Reference
Lesion fluid, crust material, or scab	N/A	Sterile tube/container	[13]
Swab of lesion fluid	N/A	Viral transport media	[13]
Nasopharyngeal and/or throat swab (useful in febrile patients)	N/A	Viral transport media	[14]
Blood serum (useful in febrile patients)	≥0.5 mL	Serum separator tubes	[15]
Cerebrospinal fluid (CSF) (useful in encephalitic patients)	≥0.5 mL	Sterile tube /container	[16]
Urine (on severly ill patients)	50 mL	Sterile tube /container	[17]
Frozen tissues/formalin-fixed, or paraffin embedded tissues	N/A	Sterile container	[18]

previously given to population, may interfere with serological testing.[19] However, from recently ill patients, it has been reported that IgM and IgG can aid diagnosis if PCR result test is inconclusive.[16]

2.2. Physiochemical Features of MPXV and Its Importance in Current Diagnosis

The chemical and physical features of a virus are extremely informative to develop not only sensing technology but also to choose appropriate nanoscale physio-chemical characterization tools such as atomic force microscope (discussed in detailed later) to elucidate several important features of the virus. These features include understanding nanomechanical properties of the virus with host cells or to understand the interaction of virus with potential drug molecules. **Figure 2**a–c shows electron microgram of MPXV which show that it is a 200–250 nm brick-shaped virus with surface tubules and dumbell-shaped core components. From chemical perspective the genome of MPXV is composed of double-stranded (ds) deoxyribonucleic acid (DNA).

From diagnosis perspective double stranded feature of MPXV has its pros and cons as compared to SARS-CoV-2 virus which is a single stranded RNA virus. Essentially, the DNA viruses first convert their DNA to RNA before making viral proteins in the host, unlike RNA virus which may directly start to express proteins once they enter the cell.[22] This means that virus may stay longer in the body before showing obvious symptoms in the infected individual. This will lead to unnoticed spread of virus from infected individual in the community which may appear at a latter to an individual who got infected via non-contact human-to-human transmission mode (such as via aerosol). This is apparently one reason why the virus has spread in many geographical locations undetected, representing a challenge for biosensors/diagnosis researchers to develop new technologies which can detect MPXV at an early stage. However, the good news is that the DNA viruses are relatively easier and more accurate to detect compared to the RNA viruses using PCR techniques. This is because the DNA virus detection does not require reverse transcription before PCR, thereby ensuring that the target of interest (which is dsDNA) is relatively stable compared to single stranded RNA.

3. Biosensors

Traditionally, biosensors constitute devices which measure chemical or biological reactions by generating signals proportional to the concentration of target analyte in the reaction. While this concept has not changed in the recent times, several other features such as artificial intelligence, data analysis, and advanced real-time signal readouts have been added to the biosensor systems which were once primarily limited to transducers and biorecognition layers. This has provided an opportunity to fast-track the translation of biosensors to the market in a short duration of time. There is a vast literature on different types of such biosensors developed in the recent times. However, here we will focus on biosensors in the context of early detection of MPXV while shedding light on the different types of biosensors which are more suitable for early detection of MPXV.

3.1. Biorecognition Routes

We identify three different routes of biorecognition which are well-placed to contribute toward a development of a biosensor for the early detection of MPXV. The first route is based on the "direct recognition" of the whole virus[23] while the second route comprises "semi-direct recognition" methods of virus detection via identification of circulating DNA or recognition of the viral surface protein in the body tissues.[24] Last methodology is categorized under "indirect recognition" which mainly comprises serological assays.[25] **Figure 3** shows examples of various bioregonition routes and **Table 2** discusses advantages and disadvantages of these methods compared to each other.

3.1.1. Direct Recognition Methods

In direct method, the whole intact virus or fragments of virus are detected in the sample collected from the suspected individual. The virus recognition event is achieved with the help of antibodies specific to the surface proteins of the virus. In the case of MPXV, there are several proteins and lipid molecules in the virus envelope which aid in its binding to the

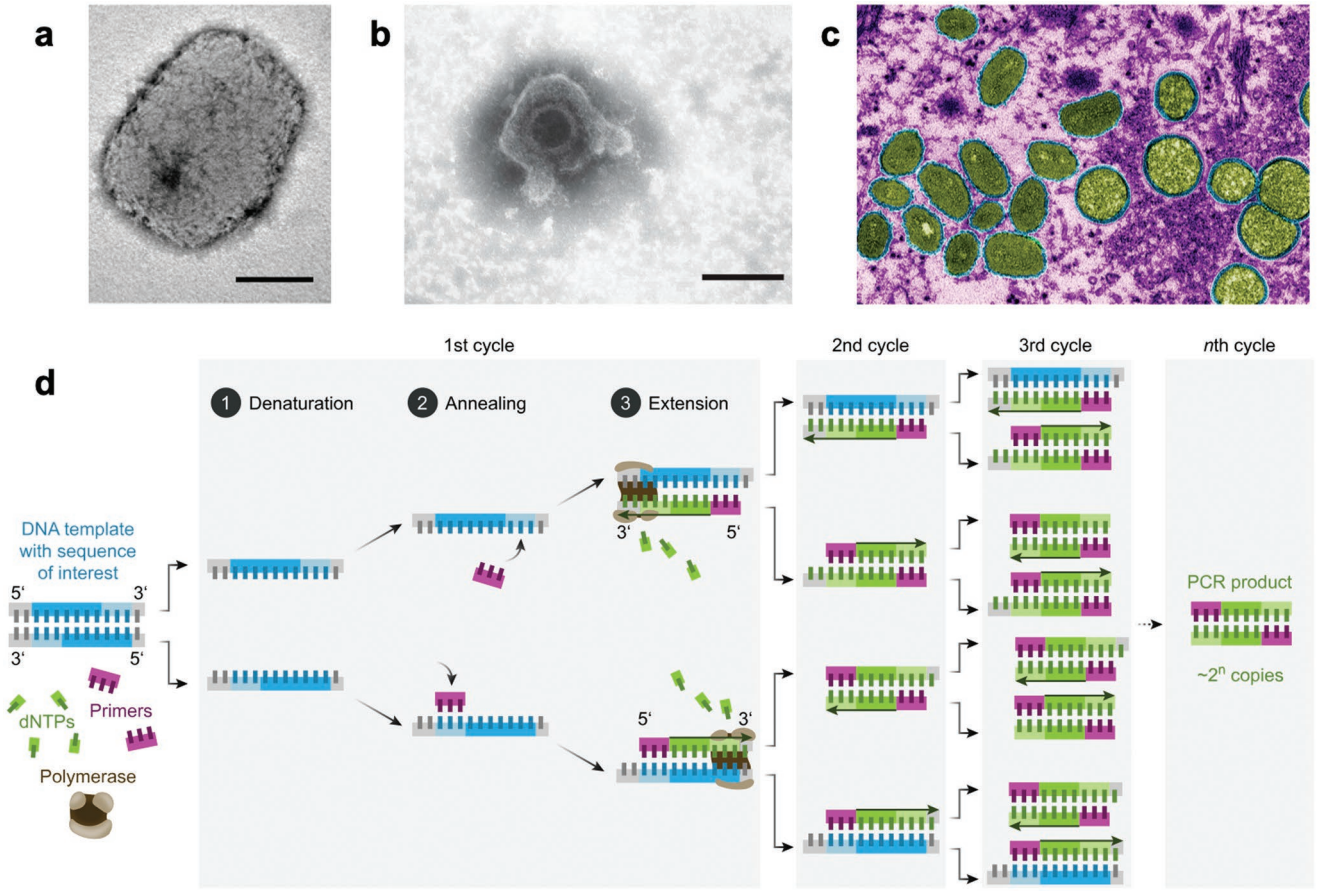

Figure 2. Monkeyvirus and conventional detection test: a,b) electron microscopy image of a single monkeyvirus (MPXV). The scale bar in the image (a,b) is 200 nm. c) Colored electron microgram of MPXV and d) steps of polymerase chain reaction (PCR), which is currently the preferred technique to detect the double stranded DNA of the MPXV. Image (a) reproduced under the terms of the CC-BY license.[9] Copyright 2019, Elsevier, and image (b) reproduced under the terms of the CC-BY license.[20] Copyright 2018, MDPI. Image (c) is adapted with permission.[21] Copyright 2022, American Associate for the Advancement of Science and image (d) is reproduced with permission under the CC-BY-SA 4.0 license: https://en.wikipedia.org/wiki/Polymerase_chain_reaction#/media/File:Polymerase_chain_reaction-en.svg.

host cell's glycosaminoglycans,[38] see Figure 3a. The virus also consists of surface tubules for which synthetic antibodies can be constructed to assist in direct detection of the virus.[39] For instance, molecular imprinted polymers (MIPs) can be used to develop a mold which will attach the virus of interest. Molecular imprinting of polymers is a technique where polymers are used to create scaffolds for analyte of interest. In the process of printing, first the target analyte for which the scaffold is to be developed is immobilized on a surface. This is followed by polymerization of the chosen polymer molecule around the target analyte. The target analyte is then selectively removed leaving behind pockets which will specifically fit analyte with which the MIPs were developed. Recent reports, associated with COVID-19 disease, suggested that MIPs which are specific to a multiple variants of a virus can be developed within a time frame of 8 weeks, including its integration with transducers.[40] While detecting the whole virus is the most reliable approach to confirm a given disease, it is quite challenging detecting whole virus when less number of intact virus particles are present in the tissue or circulating in the body fluid.[29] Therefore, the use of direct detection routes may not generate the most sensitive methods for early detection of MPXV in human but these can be used to develop high performance environment monitoring sensors for virus. For instance, to detect MPXV in sewage waste or for monitoring virus particles in air (discussed in later sections), the direct virus detection is desirable as viruses mainly remain in their intact state in such environments.[30]

3.1.2. Semi-Direct Recognition Methods

Recognition by a DNA primer, hereafter referred to "nanoPCR," which utilizes nanoscale transducers to facilitate conventional PCR constitute one of the main semi-direct recognition method.[27] The nanoPCR, see Figure 3b, provides advantages of higher sensitivity and specificity toward the target, low manufacturing cost, portability, and possibility of translation of biosensors to easy-to-use consumer friendly sensors formats (e.g., a sensor which can be self-used upon purchase from supermarket).[41,42] Other main semi-direct methods include

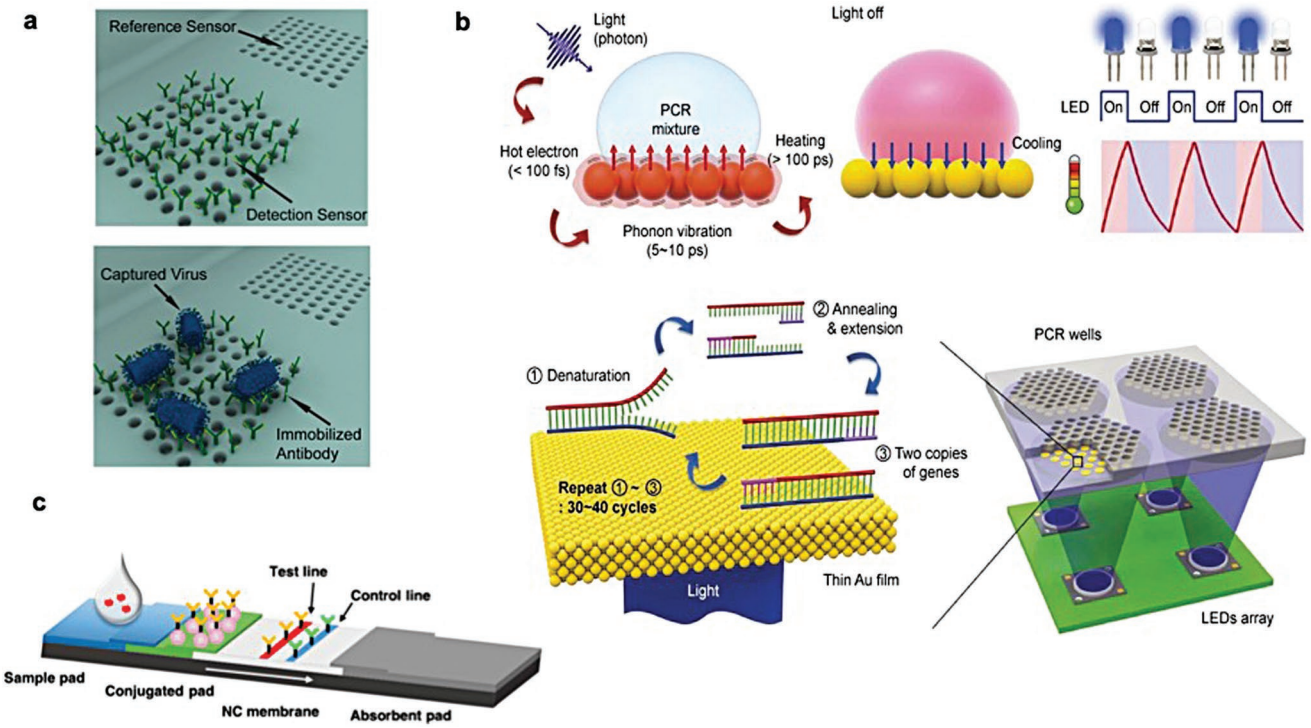

Figure 3. Routes for MPXV detection: a) direct detection of whole MPXV using plasmonic nanoholes. Reproduced with permission.[26] Copyright 2010, American Chemical Society; b) semi-direct detection of virus using nanoPCR involving nanoplasmonic transduction technology. Reproduced under the terms of the CCBY license.[27] Copyright 2015, Springer Nature. c) An example of an indirect serelogical test utilizing lateral flow biosensing devices. Reproduced under the terms of the CC-BY license.[28] Copyright 2021, MPDI.

detection of surface proteins of virus within body tissues such as blood or a biopsy sample extracted from lesions.[43]

Other routes include detection of MPXV dsDNA or fragments using bioassays.[31] New methods based on detection of surface proteins using monoclonal antibodies or synthetic binders such as MIPs can also be developed for the detection of the MPXV.[32] The major advantage of semi-direct methods is its capacity to detect viral DNA and proteins in low number. Several nanosensors have been developed in the recent decades which can detect amino acid and nucleic acid based biomolecues down to zepto molar concentrations in blood with high specificity.[33,44] Therefore, semi-direct methods serve as an ideal route to detect MPXV infections at an early stage, when the viral load in the body is low.

3.1.3. Indirect Methods

The indirect detection is primarily based on identification of biomarkers associated with the monkeypox disease. For instance, detection of MPXV antibodies or detection of IgG and IgM in the blood aids to identify the MPXV disease.[45] The main advantage of the indirect method is the fact that it is easy and quick to perform which leads to development of several advanced bioassays kits currently available even for home use (such as lateral flow devices, Figure 3c) capable to detect antibodies with high sensitivity and selectivity.[34] However, due to the fact that the world population has been vaccinated for smallpox virus (another category of orthopoxvirus), there is a high cross reactivity of the smallpox antibodies present in

Table 2. Biorecognition methods.

	Pros	Cons	Reference
Direct	High specificity as target of interest is directly detected. High relaibility as specific target of interest can be quantified. Useful for detection in evnironments where viruses remains intact alogside other homologous viruses (such as sewage waste).	Low-sensitivity. Requires sensor to comply with higher levels of safety requirements (in case where sensor is detecting the whole virus).	[27, 29, 30]
Semi-direct	Higher sensitivity than the direct methods. Compared to direct methods, semi-direct methods are easier to translate in easy to use consumer friendly portable sensor formats.	Lower specificty than the direct methods and at times, prone to false positive and false negative results.	[31–33]
Indirect	Easy to tune the sensor response as signal amplification can be enhanced by coupling a secondary reagent.	Low cross reactivity. Use of additional reagents may increase the cost and assay time.	[34–37]

the blood which limits use of serological assays as standalone detection techniques. Similarly, there are several other infections[35,36] where levels of IgG and IgM are altered in the body and therefore indirect detection methods for MPXV should only be used as complementary detection tools, in cases where the PCR is inconclusive due to the low levels of MPXV dsDNA. This is in contrast to the success of serological assay kits used for the detection of COVID-19, where antibody tests were also used to confirm the presence of the disease in an individual, especially to check if the individual was recently infected.[37]

3.2. Transducers and Point-Of-Care Systems for MPXV Detection

Transduction techniques, based on the principle of electrochemistry or optics have been vastly developed in academic and enjoyed success in translation of biosensors to industry.[46,47] While limited work has been accomplished on the MPXV detection, there are many examples of vaccinia virus detection which is often considered as surrogate for MPXV.[48] For example Donaldson et al., developed a fiber optic based sensor combined with fluorescent bioassay for rapid detection of vaccinia virus from throat swab specimen.[49] The authors reported 2.5×10^5 pfu mL^{-1} as their limit of detection. In another example optofluidic nanoplasmonic biosensor was utilized to detect MPXV using antibodies immobilized on plasmonic nanoholes with a limit of detection of around 10^8 pfu mL^{-1}.[26] Researchers have also developed advanced fluorescent assays which use antimicrobial peptides to directly capture vaccinia virus, with a sensitivity of 5×10^5 pfu mL^{-1}, that is slightly higher than antibodies.[50] Most of these sensors achieved binding with 15–30 min suggesting that sensitive detection of MPXV is also achievable within few minutes of sample collection. Another biosensor technology based on the principle of quartz crystal microbalance (QCM) has been utilized to even detect airborne vaccinia virus with a limit of detection between 8.5×10^8 to 8.5×10^{10} pfu mL^{-1}.[51] The applicability of the QCM based sensors in air for the detection of vaccinia opens several opportunities to develop MPXV biosensors for use in areas of large public gatherings. Among other electrochemical based technologies, voltammetry-based transduction methods have been developed for the detection of vaccinia virus in the presence of L-ascorbic acid with a detection limit of 4×10^3 pfu mL^{-1}.[52] Among the few reports on MPXV detection, microarray based technology which relies on printing of oligonucleotide probes to a single spot has been proposed by Kostona et al. for early detection of monkeypox disease.[53]

While these technologies are novel, the main challenges appear in form of ability to ensure cost-effectiveness, easy to use, long shelf life of the sensor, and feasibility for mass manufacturing of the biosensor.[54] In this respect, several efforts have been made by industrial sector in the last couple of months as a result of which we now have monkeypox antigen rapid test kit (from companies such as Joysbio, China, and VivaChek, USA) which can provide results with 15–20 min including sample collection, see **Figure 4** for details. These kits work on the principle of lateral flow assay system and provide a qualitative result for the presence or absence of MPXV via color change of test and control lines within the flow strip. It should be noted that both antigen and antibody tests for MPXV are available in lateral flow device formats, where former works by detection of virus antigen from skin lesions and the later detects IgG/IgM antibodies in finger prick blood.

There are also several opportunities for point of care devices to be used exclusively for rapid detection of MPXV either for personal use in home (which can be purchased in the form of consumer electronic device from a retail shop) or at the doctor's workbench. From technical perspective the most significant challenge will be to address the specificity of the sensor that is, to specifically detect MPXV in the presence of other bacteria and virus. Integration of sensing and imaging techniques in the single platform could be one possible strategy to allow specific detection of MPXV. For example Wei et al. have developed a cell phone based imaging to detect individual viruses using an optomechanical attachment.[55] There are also examples of advanced sensors which use smartphone as analyzers and display unit for rapid, real-time, and point-of-care monitoring of the target analyte.[56,57] The sensing principles are mainly based on fluorescence and colorimetric detection mechanisms while the imaging systems are based on brightfield microscopy and imaging, showdown imaging, and lens-free microscopy.

3.3. Early-Warning Community Sensors

Early warning community (EWC) sensors represents sensing systems which have the capacity to generate and disseminate timely and meaningful warning information that enables communities, at-risk individuals, and organizations to prepare and act appropriately and in sufficient time to reduce harm or loss.[54] The fact that MPXV has silently spread across several nationals, there is a pressing need to develop community based early warning systems. In this respect, we identify four environments (sewage, air, soil, and food) where detection of MPXV can contain community level spread of the virus. See **Figure 5** describing possible routes of MPXV leading to their presence in the above environments.

Among these environments the detection of virus in air is extremely challenging.[58] This is due to the possibility of non-specific attachment of other virus shaped particles from the air including dust or other micro-organisms. These are unlike the liquid-use biosensors where there is a possibility of washing the sensor to remove the non-specifically attached micro/nano entities from the surface.[59] However, these are extremely useful sensors to contain viruses in community. For example, such sensors are ideal for installation in the classrooms of schools, colleges, universities, or even in working offices of any organization,[60] where they can continuously monitor the air quality and warn the occupants of a potential presence of a virus. One can also install such sensors in transport means such as airplanes, trains, and buses as travel is one of the causes of any geographical spread of viruses, let alone be MPXV. Recently cases of MPXV have been found in air samples collected before and during bedding change in the patient room, suggesting that indoor air monitoring biosensors for MPXV are required to counter silent spread of MPXV.[60,61]

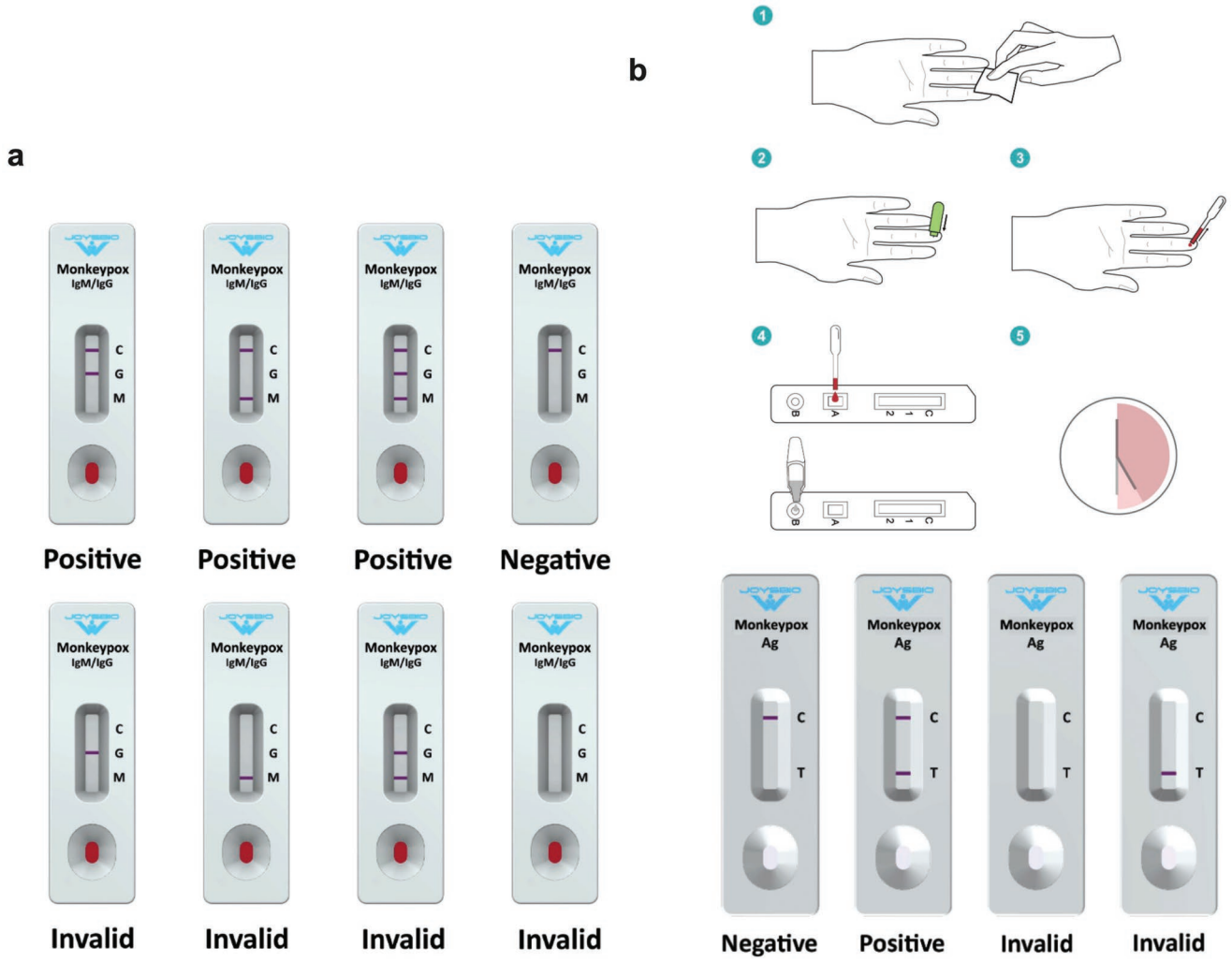

Figure 4. Biosensors for rapid MPXV detection: a) shows commercially available monkeypox test based on the detection of antibodies. b) Steps in sample collection from a finger prick blood and subsequent analysis using antigen test on lateral flow device. These images are used in this work with permission from JoysBio company.

Drinking water and food are two more sources which are directly consumed by humans. MPXV cases in humans, currently present in some geographical locations, with no or limited travel history might be associated with consumption of contaminated water and food especially, in Europe/UK where many food items are exported from African continent.[62] Several sensors have been developed in the past for detection of viruses in food and drinking water which can be re-purposed for the detection of MPXV.[63,64] It should be noted that sensors developed for detection of MPXV in soil can perhaps limit the spread of the virus to food and drinking water. The possible existence of MPXV in soil can be linked to infected water bodies, by means of sewage sludge, sewage turned manure, and irrigation water which deeply affect soil health, and with potential to eventually contaminate food which grows in it.[65]

Therefore, detection of MPXV in sewage plants or sewage discharge areas may also lead to early detection of monkeypox disease. Humans can shed viruses in large amounts in feces for days or weeks, both before and after onset of symptoms which will allow early detection of both symptomatic and asymptomatic individuals.[66] Additionally, such systems may also assist in understanding the spread of the monkeypox disease by correlating the geographical presence of the virus in different areas. One opportunity to enable such sensing strategy is to utilize artificial intelligent (AI) systems and make evidence based decision for public health intervention, as also shown in **Figure 6**. From the perspective of measurement, the main benefit of the AI based biosensing is to reduce noise and enhance specificity of detection.[67] For instance, in the case of MPXV, effective machine learning algorithms (MLA) can help in specific identification of MPXV given its overlap of symptoms with other viruses at initial stages of the infections. Similarly, in the later stages, it is difficult to specifically detect MPXV given its resemblance to smallpox virus. Several MLA approaches based on classification, regression, dimensional reduction, feature extraction, image recognition, and clustering can be exploited to discover specific hidden relationships between the sensing signals and sample parameters.[68] Such attributes will also

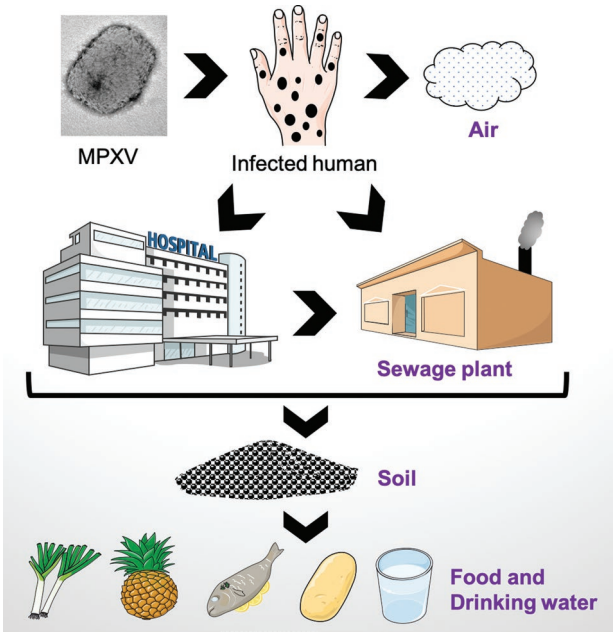

Figure 5. Community map for MPXV detection. The figure shows a link between various elements of the environments. First the virus can infect the human being which can then be easily transferred to air or sewage plants/hospital waste. As most of the waste is disposed of in the earth, the MPXV has a potential to reach the soil. The soil then further contaminates the food which eventually has potential to infect human and animal life. Within this context, this schematic emphasis the areas where detection of MPXV is essentially as a first step to cut its transmission paths in the environment.

assist biosensors to provide rapid readout of the signals which is a significant requirement for on-site detection or diagnosis of MPXV in areas susceptible to transmission of MPXV, such as in the classrooms, offices, malls, sports arena, cinema, restaurants, and border control areas, etc.

3.4. Opportunities and Challenges for State-Of-Art Biosensing Technologies

Apart from the afore discussed sensor technology and biorecognition strategies, several newly developed biosensing technologies/transduction principles can be extended/developed for MPXV detection. For instance, aptamer based biorecognition layers for specific detection of MPXV can be developed and integrated with a range of electronic and optical transducers transductions.[70–72] Some other types of biorecognition elements which can be developed include fragment antigen-binding, single chain variable fragments, and the non-covalent variable fragments in form diabodies and tribodies.[73–76] Most of these bioentities are currently not available for MPXV detection. This is due to the fact that identification of specific antigens or target biomarkers are required for MPXV. Once identified, several biomolecular techniques can be used to develop, even to scale up at commercial level, to a level where these bioentities can be incorporated in the multiplexed biosensing system. Here we refer to multiplexed systems as those sensing formats where more than one analyte specific to MPXV can be detected in a single biosample (such as blood, urine, saliva). Recently, several optical and electrochemical transducers have been developed for virus detection with high sensitivity. Most of these can easily be repurposed for MPXV detection. For example, potentiometric sensor for detection of influenza virus and SARS-CoV-2 was recently developed by Lee et al. with limit of detection less than 200 PFU mL^{-1} for both viruses.[77] In another study, Gomez

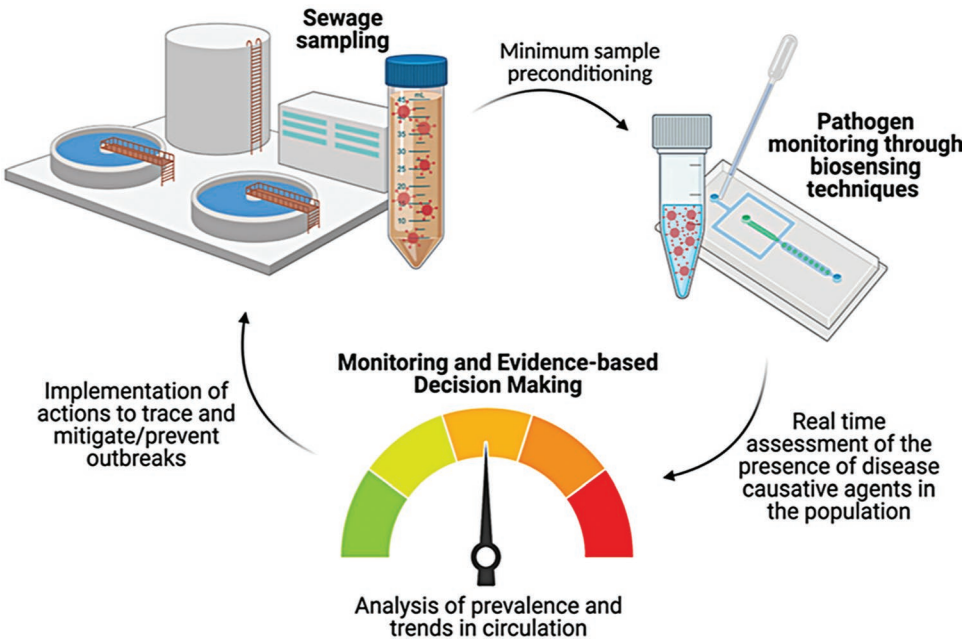

Figure 6. Biosensors for decision-making: here we show how biosensors can be used to make evidence-based strategies and decisions for the monitoring of viral pathogens (such as MPXV) in wastewater with an overall aim of mitigating, tracing, and preventing MPXV outbreaks in resource-limited regions. Reproduced with permission.[69] Copyright 2022, Elsevier.

et al. developed a microfluidic photonic integrated circuit based biosensor for detection of six viruses: classical swine fever virus (CSFV), African swine fever virus (ASFV), porcine porcine parvovirus (PPV), porcine circovirus 2 (PCV2), a reproductive and respiratory syndrome virus (PPRSV), swine influenza virus A (SIV).[78] Note that the limit of detection of individual viruses is not investigated in this work. The detection of viruses have also been recently achieved in a completely label-free manner using other techniques including localized surface plasmon resonance (limit of detection 319 copies mL^{-1}),[79] time-resolved luminescence (limit of detection 4.3×10^4 PFU mL^{-1})[80] and many others discussed in recent literature on technologies for virus detection and analysis.[81–85] However, the most significant challenge is the quick translation of this technology for addressing MPXV spread. Strong collaboration between industry and academia, large scale investment by funding agencies and compliance of the technology with ethics and government regulation will help fast transition of biosensing technology developed in laboratory to industry.

4. Nanoparticles as Analytical Tool for MPXV

Emergence of nanotechnology lead to the growth of using nanomaterials in medical science and research. Nanomaterials are described as materials with a single dimensional feature with the size between 1 and 100 nm. Nanoparticles (NPs) possess physical and chemical properties which are superior to their existence in bulk forms due to their high surface area, modifiable surfaces and controllable lipophilicity/hydrophobicity.[86] These unique properties enable incorporation of multiple antiviral agents onto the same nanoparticle. Due to the mentioned significant properties, their application in biology and medical science has also led to recent advances and discoveries in biosensing, imaging, drug delivery, cancer therapeutics, and antiviral agents.[87,88] In a viral infection, multivalent interactions of virus and the host cell surface leads to intake of the viral particle through different mechanisms such as cell fusion and endocytosis. Therefore, inhibiting the cell-pathogen interactions can avoid subsequent infection caused by viruses.

According to the ingredients of nanoparticles structure, nanoparticles are classified into organic and inorganic categories.[86] The principal mechanism of antivirals nanoparticles is based on suppressing viral reproduction in host cells, neutralization of virus, and prevents the binding of virus to target cells. The most common organic NPs used for antiviral applications are polymeric NPs, dendrimers, micelles, and lipid-based NPs. The most common inorganic NPs employed for treating the viral infection are gold, silver, copper, and aluminum NPs, metal oxides such as iron oxide, magnesium oxide, titanium oxide, and zinc oxide NPs, semiconductors such as silicon and ceramics as well as graphene and quantum dots. Table in Supporting Information summarizes recent applications and mechanism of NPs in antiviral functionality. Due to the smaller size, improved constancy, activated discharging, enhanced penetrance, tunable adjustability, more stability, and withstanding against harsh conditions, the non-organics NPs attract more attention.

Recent studies demonstrate using metal NPs such as silver or gold can reduce the infectivity of the viral cultured cells.[89–91] This is due to the antiviral activity of metal NPs via direct interaction between NPs and viral surface proteins. So far, use of metal NPs has been exploited as antiviral agents against respiratory syncytial virus,[92] herpes simplex virus type 1,[93] hepatitis B virus,[94] tacaribe virus,[95] influenza virus,[96] and MPXV.[97] Rogers et al.[97] have synthesized and employed silver NPs (Ag NPs), polysaccharide coated Ag NPs as well as silver nitrate ($AgNO_3$) with different sizes from 10 to 80 nm to inhibit the infection of monkeypox. These NPs have been produced via plasma gas synthesis and have been evaluated in different concentration from 12.5 to 100 mg mL^{-1} to inhibit infectivity of MPXV virus using a plaque reduction assay by blocking the virus—host cell binding and penetration, making them inefficient for replication. They demonstrated Ag-NP-55 and Ag-PS-25 exhibited a significant dose-dependent inhibition of MPXV plaque formation. They also reported that except for the concentration of 100 μg mL^{-1} $AgNO_3$, there was not any cytotoxicity (Vero cell monolayer sloughing). However, the underlying mechanism of inhibition remains elusive and need further studies.[90]

Ziem et al.,[98] have synthesized and functionalized the highly efficient multivalent 2D flexible carbon architecture to inhibit othopoxvirus viruses. The concept was based on the utilization of polysulfation to mimic the heparan sulfate-containing surface of cells which competed with the binding site of the viruses. This strategy resulted in highly efficient inhibition of orthopoxvirus infection. Hence, this finding promises development of potent inhibitors for orthopoxvirus viruses including MPXV which own a heparan sulfate-dependent cell entry mechanism. Moreover, recently researchers are working on development of lipid nanoparticle (LNPs) formulation as an organic nanoparticle to increase efficiency of DNA-vectored vaccines/immunoprophylaxis.[99] The main advantage of using LNPs in comparison with viral vectors is the absence of immunogenic viral proteins. These studies pave the way to use NPs and LNPs in vaccine and drug development to fight against viruses including MPXV. However, despite significant enhancement in nanomaterials technology used for antiviral applications including MPXV, there are several challenges which should be considered. The biocompatibility of nanoparticles is one of the main factors which needs to be improved as the efficacy of antiviral drugs would be decreased due to the dissolubility of nanoparticles. It can be improved by designing effective synthesis procedure. As the targeting specific virus by nanoparticles is an essential task, enhancing the specificity of nanoparticles-based antiviral drugs is a key factor. As mentioned, the principal mechanism of functional nanoparticles is still largely unknown which required more systematic study to discover. Furthermore, in vivo delivering nanoparticles into the cells and tissue needs more effort as the ultimate goal is using the antiviral agents in clinical trials. Finally, nanoparticle toxicity is an important issue which should be constantly considered which needs to perform more research on the stability and degradation of functional nanoparticles used for MPXV and other antiviral applications.

5. Bio-Nano for MPXV Vaccine Development

In this section, we survey the re-purposing of the potential smallpox vaccines and provide an outlook of vaccine development for MPXV considering the benefits and risks. At this time, there are no distinct treatments or vaccine available for monkeypox infection, however, recent research demonstrates that the licensed smallpox vaccines can be used to control a monkeypox outbreak.[100] Nowadays, replication-component and replication-deficient smallpox vaccines are most commonly used vaccines used to avoid the smallpox and monkeypox infections spread. Recent analysis demonstrates the smallpox vaccines have more than 85% effectiveness against monkeypox infection.[101] The smallpox vaccine used nowadays mostly consists of live vaccinia virus (VACV). Vaccinia is a double-stranded DNA virus with a wide host range belonging to the genus orthopoxvirus along with variola virus. The origin of vaccinia is uncertain, and there are numerous variants of vaccinia with different biological properties.[102] Vaccinia induces both cellular and humoral immunity to variola virus and can efficiently resist infection of other poxviruses such as smallpox, vaccinia, and monkeypox. The underpinning reasons of efficiency of VACV vaccines for both MPXV and variola virus is due to the high similarity in the most of the proteins of these viruses.[103,104] Historically, development of VACV vaccines consists of four generations. In the first generation, the live animals were utilized to growth the different vaccines. To control microbial contamination, standardize the vaccine development procedures and prevent allergenic sensibilization to animal proteins exploited in vaccine, in the second generation of VACV vaccine development, the culture systems or embryonated chicken eggs have been employed.[105] Due to the undesirable safety of VACV second-generation vaccines, multiple immense passages of a parental vaccine strain in cultured cells has been proposed as third generation VACV vaccines.[105] This strategy attenuates VACV via production of random deletions and mutations. Finally, the advent of genetic engineering provides an opportunity to develop fourth generation of VACV vaccines via insertion or deletion of specific genes in the orthopoxvirus genome.[105] In this case, the VACV can be amended to be utilized as an expression vector system as foreign DNA could be placed into peripheral areas of VACV genomic sequences.

Due to the presence of live vaccinia virus in replication-competent vaccines, there is a risk of replication in human cell which may cause serious effects, lesions, and spreading. Due to the complications of vaccinia virus, it is recommended that people with severe immunodeficiency should avoid smallpox vaccination. As the risk of side effects in local contact with the virus is anticipated to be similar as the recipient of the vaccine, so, special care should be paid to the vaccination area to avoid the transmission of the virus.

However, although the smallpox vaccines based on VACV are highly efficient, there are possibilities of serious side effects especially for individuals who have history of eczema or are immunocompromised. Hence, development of more secure live vaccines like modified VACV Ankara (MVA) was proposed. MVA had been extracted using 500 passages of the parent virus in chicken embryo fibroblast cells,[106] cause the mutations and deletions which critically confine the replication and virulence of the vaccine even in immuno-compromised hosts.[106] The studies depict priming and boosting by MVA could provide more than 2 years protection in a MPXV challenge model.[107] Moreover, the accurate immune mechanisms that smallpox vaccine elicits immunity to monkeypox remain unexplored. Therefore, to develop and test the next generation of safer smallpox vaccines as well as novel monkeypox vaccine, it is essential to identify protective and problematic immunogens. It can be performed by understanding further knowledge of orthopoxvirus proteins, the infection mechanisms, and their association with immunity. The possible suggestions are development of attenuated variants of vaccinia virus, recombinant proteins, and vectors. Henceforth, considering the recent increase in MPXV, it is mandated and highly required to develop both DNA vaccines and subunit vaccines specifically designed for MPXV to prevent its outbreak. To summarize the recent vaccination possibility for MPVX, **Table 3** provides a summary, the pros and cons of antiviral agents that can be used to inhibit monkeypox infection.

6. Nanoscale Visualization and Characterization Tools for MPXV

Spectroscopy, diffraction, and microscopy are the main nanoscale approaches that have been used for direct diagnosis,

Table 3. Potential antiviral agents for MPXV inhibition.

Name	Dose	Mechanism	Advantages	Disadvantages
ACAM2000	A single dose of 0.0025 mL of vaccinia virus (live) consists of $2.5–12.5 \times 10^5$ plaque forming units per dose.	Vaccinia virus cross-protects against variola virus induced immunity.	Generally safe and effective. Recombinant vaccinia viruses. There is no variola virus in vaccine so cannot cause smallpox.	Can replicate in human cell. The risk of side effects in domestic contacts is same as the vaccine recipient. High-risk for immunodeficiency population groups.
APSV	A single dose of 0.0025 mL of vaccinia virus (live) consists of $2.5–12.5 \times 10^5$ plaque forming units per dose.	Vaccinia virus cross-protects against variola virus induced immunity.	Generally safe and effective. There is no variola virus in vaccine so cannot cause smallpox.	Can replicate in human cell. The risk of side effects in domestic contacts is same as the vaccine recipient. Probability of severe adverse reactions.
MVA	Two doses (0.5 mL each) separated by 4 weeks for non-vaccinated individuals. One dose (0.5 mL) for previously vaccinated individuals.	Humoral and cellular immune responses to orthopoxviruses.	Live, attenuated, replication-deficient vaccine. Can be employed for immune deficiencies individuals. Less side effects. No severe adverse events.	Need more study by adequate human studies. It is not known whether it is excreted in human milk.

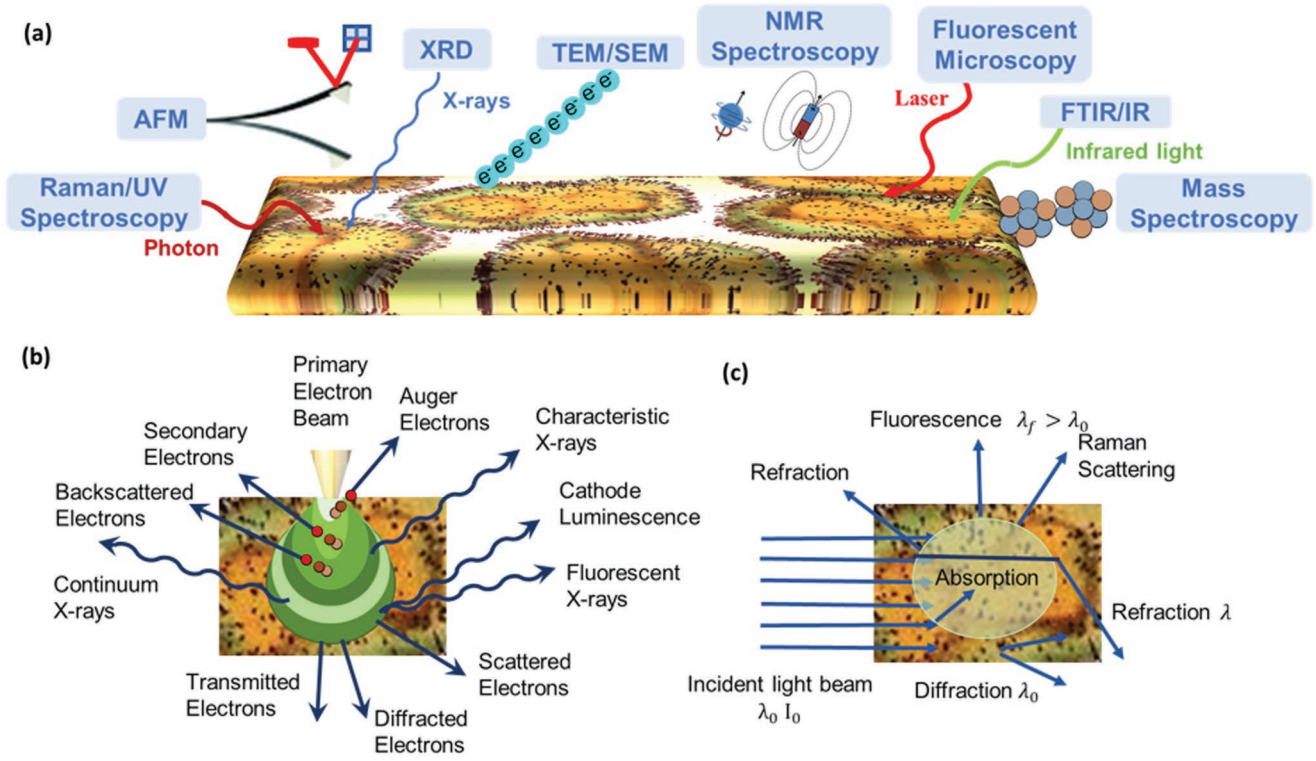

Figure 7. Visualization and characterization of MPXV: a) schematic of nanoscale analytical methods used for visualization, diagnosis, and characterization of viruses including monkeypox. b) The mechanisms of electron matter interactions and; c) the mechanism of light matter interactions.

visualization, and characterization of virus structure.[54] The exceptional abilities of these techniques provide an opportunity to not only analyze the structure, function, and properties of viruses but also investigate the viral impact on host cells, the interaction of virus with extracellular environments and nanoparticles which are highly demanded in vaccine development and drug discovery applications.[108,109] The working principle of mentioned techniques is based on light-matter, electron matter, or atom-matter interactions (**Figure 7**). **Table 4** summarizes the working principle, the resolution, advantages, and limitations of each technique.

6.1. X-Rays and Neutrons Diffraction

To identify the structure of the virus as well as visualize the macromolecular assemblies with high resolution at atomic level, X-ray diffraction (XRD) patterns are promising. The fundamental principle of XRD is based on generation of X-rays via collision of accelerated high-speed electrons in vacuum on a metal target. The produced X-rays are used to interpret the scattered X-ray intensity from crystal planes. Hitting the valence electrons of crystal atoms, leads to the diffraction of X-rays from different atomic layers. As a result, information regarding

Table 4. Comparison of high-resolution characterization techniques used in virology.

Technique	TEM	SEM	Super-resolution microscopy	AFM	XRD	NMR
Resolution	0.2–10 nm	2–10 nm	20–50 nm	≤ 1–50 nm	0.2–3.5 Å	2–3.5 Å
Advantage	Molecular structure, high magnification, conformational snapshots	Imaging surfaces at nanoscale resolution with magnification	Spatiotemporal resolution, 3D structure	Measurements in native environment, extract multiple properties	Atomic resolution	Measurements in solution
Disadvantage	Sample preparation, expensive, beam damage, dehydration	Beam damage, dehydration, metal shadowing	Bleaching, toxicity	Immobilized sample, tip artifact	Preparation time, large quantity of purified crystal	Time of acquisition, purification of molecule
Observation	Fixed samples in vacuum	Fixed samples in vacuum	Fluorescence labeled samples	Samples in ambient air or in solution	Crystal	Samples in solution
Limitations	No life process	No life process	Restricted to fluorescence labeled	Restricted to surface	Sample must be crystallizable	Need sufficiently concentraed samples

virus structure and crystallinity is collected from diffraction pattern and retrieved from detected diffraction data distributed in the reciprocal 3D space.

To determine virus structure through X-ray crystallography methods, there are five stages including purification and preparation of virus particles, crystallization, and mounting crystal particles, measuring the diffracted data, using isomorphous or molecular replacement (MR) for phase calculation and subsequently, mapping the interpretation and model construction.[110,111] Commercial sources provide the purification kits which can be utilized for preparation and purification of viruses from extracellular/tissue culture media.[112] To keep the icosahedral symmetry of the virus in the preparation stages, the handling procedure must be performed gently. Subsequently in the purification step, in order to convey the aqueous protein solution to supersaturation, the crystallization consists of nucleation and growth phases where crystal nucleus is shaped to be appropriate for molecular aggregation and after that the growth process is initiated. For virus samples, there are four techniques exploited in crystal growth including vapor diffusion, liquid–liquid diffusion, batch crystallization, and dialysis.[113] To avoid virus destruction due to the secondary radiation as well as enhance the resolution, the crystallographic data are captured at cryogenic temperature regularly about 100 K.

One of the main challenges in virus particles X-ray crystallography is growing the high-quality crystal. Conventional methods are tedious and dependent on empirical procedures. Therefore, cryo-EM/ET can be considered as supportive approach for crystallography of virus protein.[114] Integration of collected data from 3D cryo-EM with captured X-ray information could enhance the constructed model of virus particles.[115] Another type of XRD methods which exploits X-ray free-electron lasers (XFELs) is serial femtosecond crystallography (SFX).[116] Since SFX take advantage of unparalleled brilliance of XFEL beams and femtosecond pulse duration, it is desirable to investigate viruses either as single particle or crystal. Time-resolved SFX can detect the virus reaction to pH variations and the interaction between viral protein and receptors occur during life cycles of viruses.[117] Hence, integration of dynamic results of conformational variations at room temperature obtained by SFX and high-resolution static structure of viruses captured by cryo-EM can be led to generating time-resolved virus structures.[118]

Another approach to analyze the structure of viruses is neutron scattering.[119] Through this method, the quality and amount of information which can be captured from a low-resolution study of virus in solution can be improved. Using neutron scattering, the influence of different conditions in solution on the virus can be determined. Neutron diffraction is based on elastically scattering of neutrons from nuclei of the specimen.[120] Although the technique is similar to X-ray diffraction, as it generates different types of radiation, it can provide complementary information about the structure of viruses.

Utilization of X-ray and neutron crystallography approaches in the study and analysis of the family of poxviridae demonstrates the potential of the tool to analyze MPXV and its variants.[121–124] The 1.52 Å 3D-crystal structure of A42R profilin-like protein from MPXV zaire-96-I-16 is determined and explore six unique types of molecules in entry composition (**Figure 8**).[125] Matho et al.[124] described the structural characterization of D8 as one of the immunodominant VACV antigens (Figure 8a), its sequence conservation as well as its binding to the monoclonal antibody LA5, capable to neutralize VACV in the presence of complement (Figure 8b). They identified D8 sequences are almost similar in vaccinia virus, smallpox, MPXV, and other direct orthologs (Figure 8c). Gao et al.,[121] has performed XRD to solve the crystal structure of VACV E2 as the prototype member of the poxviridae, to 2.3 Å resolution(Figure 8d). Poxviridae family is the causative agent of the smallpox, and various viruse's endemic in a variety of animal species including monkeypox which can be linked with disease in humans (Figure 8). They demonstrated VACV E2 consists of C-terminal globular and N-terminal annular domains.

Matho et al.[122] have used XRD to map the A33 epitopes for A27D7 antibody and characterize the Anti-A33 antibodies. Their results suggest A27D7 is cross-species protective and can be used in neutralization of different orthpoxviruses. Furthermore, they identify that A33 is 23 kDa homodimetric type II transmembrane which undergoes O- and N- glycosylation (N125 and N135)[126] which for monkeypox the equivalent N125 site is lacked.[127] Campbell et al.[123] utilized XRD to characterize the structure of orthopoxvirus MHC class I–like protein (OMCP) that is conserved in MPXV, secreted by infected cells, and with high affinity bound by NKG2D of humans. Such structural analysis provides a ground for further progress of these compounds to antiviral drugs and inhibitors for vaccine development.

6.2. Microscopy Techniques

The main microscopy techniques exploited for virus detection and characterization can be classified as electron, atomic force, and fluorescence microscopies. Here, we briefly review the principal mechanism of each technique as well as their recent advances, challenges, and applications to study the viruses, especially MPXV.

6.2.1. Electron Microscopy

Electron microscopy (EM) is the promising high-resolution microscopy that exploits a focused beam of electrons to create images. As the electron wavelength is in orders of magnitude smaller than visible light wavelength, the resolving power of EM is higher than optical microscopes which provide an opportunity to image the structure of particles at nanoscale resolution. This capability of EM introduces it as a preeminent tool to detect, diagnose, and analyze viruses. However, using high voltage focused electron beams (between 40 and 100 kV) can damage the specimen and need intricate preparing methods. Therefore, to provide straight and homogenous electron beams, EM is always carried out in vacuum that needs to eliminate the liquids from the sample. Another advantage of EM in virus diagnosis in comparison with serological and molecular approaches, is its independence of organism-specific reagents for recognizing the pathogenic agent. For the situation of unrecognized disease or virus, although molecular tests require details of potential agents

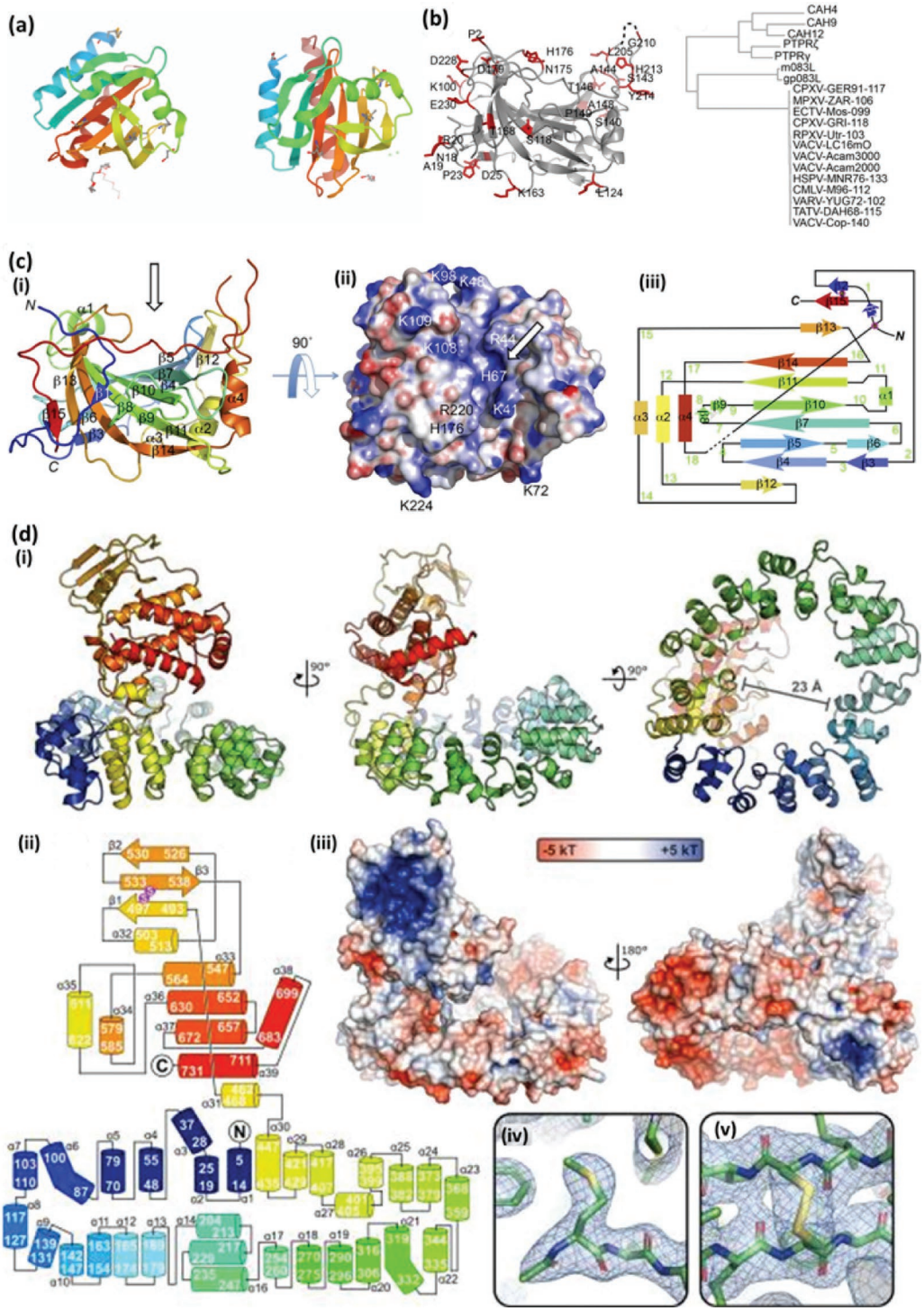

Figure 8. X-ray techniques for MPXV: a) crystals structure of A42R protein of MPXV. Adapted from Protein Data Bank, PDB structure 4QWO[125]; b) VACV D8 structure with orthopoxvirus amino acid variations highlighted in red. It also shows the dendrogram of CAH domain-containing homologs, which is captured by SeaView PhyML algorithm.[121,124] c) Crystal structure and 2D topology of D8 protein.[124]; d) structure of VACV E2 protein envelope (i), secondary schematic representation of VACV E2 (ii), molecular surface of the envelope (iii), N-acetylated initiator methionine of E2 is shown with the help of stick representation, with a refined $2F_O$-F_C electron density map (iv) and shows disulphide bond between Cys residues 496 and 535 within 2FO_FCelectron density (1.2 σ) (v). Reproduced under the terms of the CC-BY license.[121,124] Copyright 2012, American Society for Microbiology.

to identify the proper tests, EM yields an information of the elements that may be existed in a virus or specimen of interest.[128] Beside EM's potential to directly detect virus via imaging, the ultrastructure information of virus as well as its

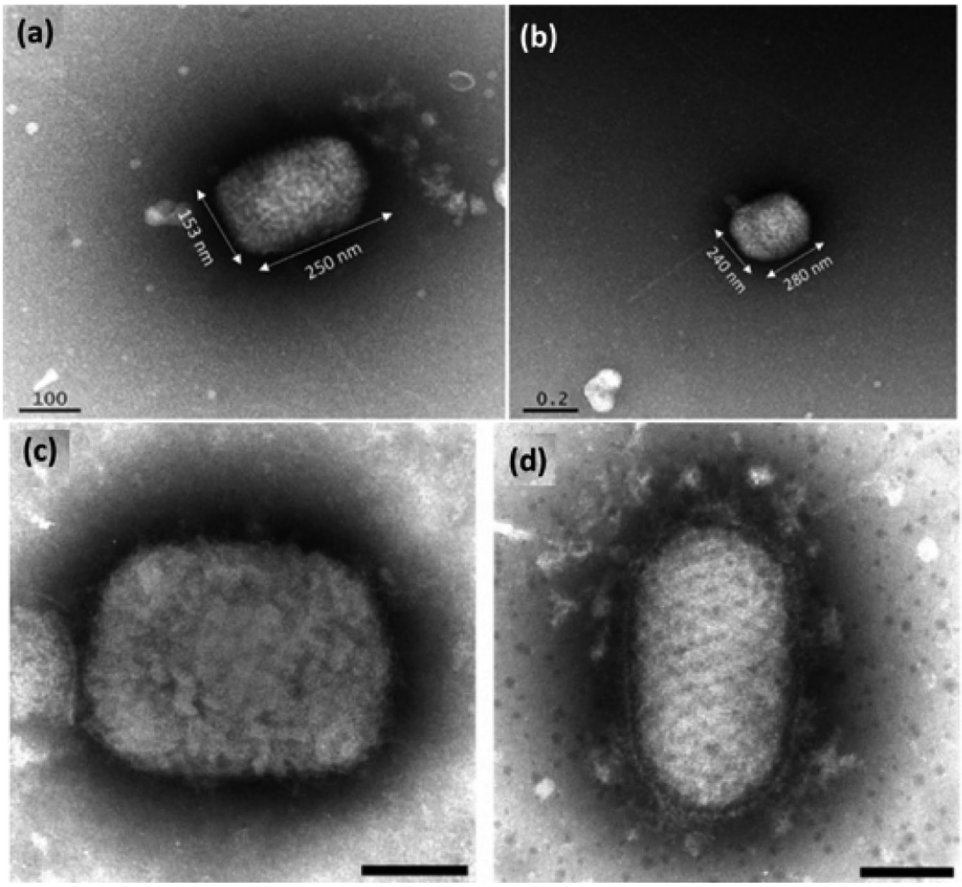

Figure 9. Electron microscopy: a,b) transmission electron microscopy of MPXV from a 38 year old patient in Singapore, 2019. The images show a brick-shaped particle, ranging from 130–290 nm. We can observe tubular structures and ring like depressions which are made visible using the phosphotungstic acid and gadolinium acetate stains; reproduced with permission.[135] Copyright 2020, Elsevier. c) Negative stain image of a clinical sample of MPXV. Scale bar: 100 nm; d) negative stain image of an orf virus. Scale bar: 100 nm; reproduced under the terms of the CC-BY license.[138] Copyright 2014, MPDI.

dynamics structure associated to the attachment and replication process can be revealed by EM which would lead to the design and discovery of antiviral agents and vaccines.[129,130]

There are several EM techniques to analyze and visualize the virus structure including transmission electron microscopy (TEM), scanning electron microscopy (SEM), cryo-electron microscopy (cryo-EM), tomography, negative staining, thin sectioning, and immune electron microscopy (IEM). TEM has been employed for narrow samples to provide 2D electron density maps of transmitted electrons. Through a series of sample rotations and collection of such images, 3D visualization of internal structures is extracted, which is called electron tomography. The basis of IEM is to form the virus immune complexes with its attributed antibody. The cryo-EM is an imaging mechanism utilized for generating 3D high-spatial resolution maps of specimen, mostly biomaterials and cells. In this approach, sequences of 2D images are acquired while it is tilted at different angles, and subsequently combined to produce 3D image.[131] SEM has been employed to quantify the virus structure.[132] The mechanism of SEM is based on capturing of diffracted electrons which gives 3D information of the surface of viruses. Integration of SEM with TEM can generate high-resolution maps of the structure of viruses, which provide comprehensive details associated to the virus characterization.[128] In the negative staining approach, through staining the background, the actual specimen would be untouched and visible. Thin sectioning is exploited to decrease the viruses into narrow layers which make them transparent to electrons.

Using EM techniques for diagnosis of orthopoxviruses including smallpox and monkeypox have been applied widely.[133,134] Fang Yong et al.[135] used transmission electron microscopy to visualize the features characteristic of orthopoxviruses (**Figure 9**a,b). Furthermore, electron microscopy and histopathology identified that poxviruses possess particular morphology—big (≈300 nm diameter), box or ovoid shape, and outer membrane protrusions (generating a textured appearance)—and reproduce in the host cells cytoplasm.[136,137] Using negative stain EM, the difference of genera orthopoxvirus and parapoxvirus has been distinguished (Figure 9c,d).[138] The results demonstrated monkeypox viruses belong to orthopoxviruses possess rectangular shape, around 225 × 300 nm in size with short and whorled filaments surface pattern. Meanwhile, parapoxviruses are oval with smaller size, approximately 150 × 200 nm, and possess a crisscross filamentous surface pattern.

However, the underlying principle of EM and XRD techniques are based on the averaging of the several particles available in the electron micrographs or crystal. Hence, collected information is limited to the single particles in a large

population. Moreover, these techniques operate at mediums that are far away from the physiological conditions of viruses and preclude the real time characterization of their dynamic properties. Therefore, atomic force microscopy can be considered as a complementary microscopy technique to image and characterize viruses in their ambient condition.

6.2.2. Atomic Force Microscopy

Since its invention, AFM has become a versatile imaging and characterization tool exploited for nanomaterials in both air and in liquid environments.[139] Compared to XRD and TEM, AFM is a quick, simple, relatively inexpensive, and more portable instrument which needed less quantity of sample for measurements. Furthermore, the AFM has the potential to be associated to another molecular approaches including PCR or electron microscopy. AFM is the nondestructive method to study the virus or its genome and provides the opportunity to detect the whole particles and determine the family, sub-family, and other complex structures of viruses. The principle of AFM performance is based on the deflection of microcantilever utilized to probe the tip-surface interaction. Depending on the type and range of interaction as well as the time scale of measurements, AFM can provide diverse range of information such as atomistic and molecular structure of the surface, physical, chemical, thermal, mechanical, electronic, and magnetic properties of specimen.[140–143] The contact mode of AFM was first invented AFM mode where the sample surface is scanned by the tip of cantilever while a constant force is inserted to the surface of specimen. For the imaging the soft materials including proteins, viruses, and other biomaterials, the inserted force should be controlled to prevent damage to the sample, undesired friction and reversible or irreversible deformation.[144] Therefore, to minimize the effect of inserted force and friction, also to provide more information from measurements, the dynamic AFM modes were invented.[145] In dynamic AFM, the microcantilever scans the sample surface while it is oscillated with the frequency equal to or close to its natural resonance frequency. There are two major operational modes in dynamic AFM, amplitude modulation (AM-AFM) and frequency modulation (FM-AFM).[146]

Due to the short contact between the tip and surface, in dynamic AFM, the friction is kept down, also the probability of damage to the tip or specimen is considerably decreased. To explore more information and properties from biomaterials like proteins, cells, tissues, and viruses and increase the spatiotemporal resolution, recently advanced AFM methods such as multifrequency, high-speed multiparametric and molecular recognition imaging modes have been developed.[147] Besides the capability of visualizing the virus particles, AFM is able to determine and measure the mechanical, chemical, and structural properties of viruses.[148] Furthermore, AFM has been employed to dissect and manipulate biological species, including viruses.[149] In order to quantify the viscoelastic, chemical, physical, and mechanical properties of viruses, force–distance curve (FDC) approach as well as multifrequency methods have been utilized as common techniques. In modern FDC, several forces versus distance curves are collected during imaging and used to map biochemical properties of the sample. Furthermore, to explore specific properties of the sample, the tip is functionalized by specific ligands. Subsequently, according to the adhesion and mechanical strength of bonds formed between receptors of sample surface and functionalized tip, the biological properties can be extracted. Applied to viruses, FDC could explore the relation between functional, structural, and mechanical properties of bacteriophages,[150,151] parvovirus minute viruses,[152] southern bean mosaic viruses,[153] and herpes simplex viruses.[154] The major challenge of FDC method is the time and high volume of data acquisition. To compensate these drawbacks and enhance the imaging speed and decrease the size of collected data, high-speed and multifrequency AFM have been developed.[147] The principle of multifrequency AFM is based on excitation and detection of different frequencies of cantilever during imaging. So, it can provide the opportunity to simultaneously image and quantify the sample of interest. Although several multifrequency AFM methods have been developed, due to the complexity in their underlying theory, theoretical studies and analysis procedures of collected data need more investigation.

The previous research on the poxviridae family by AFM methods[155] demonstrate the considerable ability of AFM to image and extract the morphological species of monkeypox. In general, to study the poxviridae family, especially MPXV, AFM can provide following information: i) determine the properties of affected cells by monkeypox; ii) explore the interplay between MPXV and host cell; iii) investigate the mechanism of MPXV to cross the cellular membrane as well as transport the monkeypox genome into the host cell and finally iv) its reproduction and characterization of the nucleic acids to comprehend the mechanism of condensing and packaging inside capsids. AFM has been used to visualize and identify the numerous integrations of proteolytic enzymes, reducing agents, and non-ionic detergents as well as the consecutive outcomes of the reactions of vaccinia virus.[156] Trindade et al.[157] demonstrated that poxviridae virus, characterized by AFM in an aqueous environment, possess a fairly regular repeating pairs of rod-shaped structures with a total width of 7–11 nm and a length of 60–90 nm. AFM techniques have been employed as a secure way to detect the vaccinia virus (VACV) in dairy cattle in Brazil[157] (**Figure** 10). As these viruses are zoonotic infections, the safe manipulation of samples is essential. Using AFM measurements in air environment, subsequent of purification and inactivation process, the relatively crude preparations of viruses have been visualized quickly. The details of efficient sample preparation are given by Trindade et al.[157]

Moreover, AFM measurements of the vaccinia virus revealed the lipid membranes, ordered and disordered protein shells, as well as nucleic acid within the vaccinia virus[158–160] (Figure 10b). The results identify a double stranded DNA genome bound by several protein shells. It also has associated with its inner core two unusual protein assemblies known as lateral bodies.

6.2.3. Fluorescent Microscopy

Fluorescence microscopy is an imaging method in light microscopes that permits the excitation of fluorophores and subsequent detection of the fluorescence signal. The implementation of fluorescence optical microscopy to visualize the

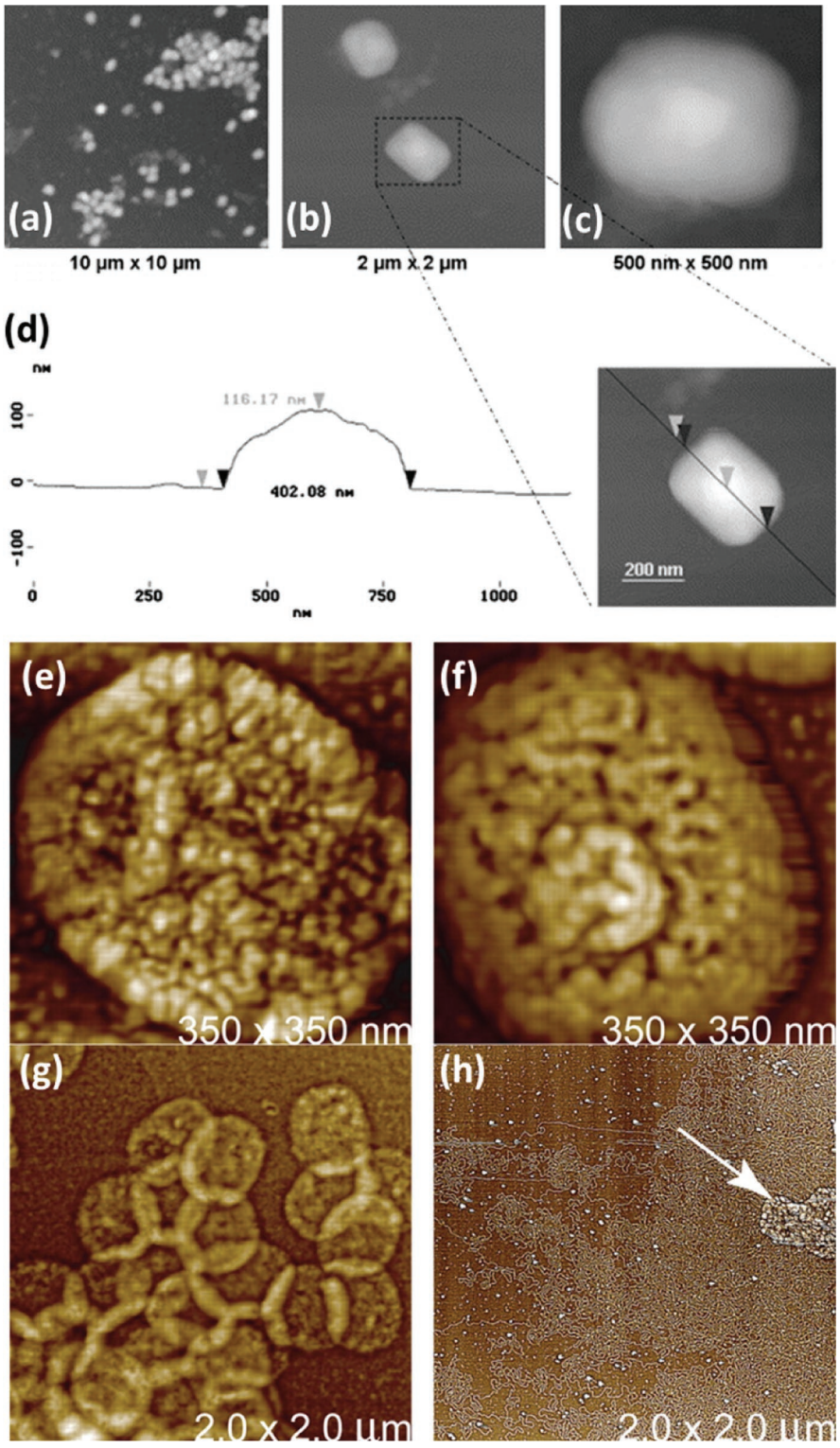

Figure 10. AFM for MPXV: a–c) AFM image of purified MV OPXV particles adsorbed on mica; d) topographical profile (left) which demonstrates virus dimensions. The arrow indicates a disrupted vaccinia virus core adjacent to the DNA. Reproduced with permission.[157] Copyright 2007, Elsevier. e) An intact vaccinia virus virion; f) the image of the virus core obtained after the virus sample with non-ionic detergent (NP-40) and dithiothreitol. g) The ghosts, or capsules, that remain after the virus cores have been treated with proteases for releasing the viral nucleic acid; h) DNA released onto the AFM substrate by disrupted viral cores. The arrow shows a disrupted vaccinia virus core adjacent to the DNA. Reproduced with permission.[156] Copyright 2013, American Society for Microbiology.

virus structures and characterize its morphology possesses several advantages, such as high specificity by using specialized fluorescent probes, 3D imaging, multicolored characterization, and live-cell compatibility. However, the major limitation of fluorescence optical microscopy is its physical limitations in optics, mainly diffraction limit.[161] The diffraction limitation makes it more difficult to study and image the nanoscale macromolecules such as viruses.[162] Recently, several technological developments have been performed to enhance the functionality, resolution, and sensitivity of light microscopes,[163] especially the invention of super-resolution microscopy (SRM). SRM methods utilize an extensive variety of chemical or physical approaches to overcome the diffraction limit of optical microscopy and pave the way for direct visualization of virus particles. Using SRM, it is possible to investigate the virus–host interactions, viral replication, and transmission as well as the characterization of anti-viral agents and vaccines.[163,164] Stimulated emission depletion (STED) is a laser confocal scanning microscopy method that illuminates the sample by two aligned laser beams. The first one is a common excitation beam that stimulates fluorophores and the second one is a depletive doughnut shaped laser that smooths the fluorescence signal around the excitation scanning point.[165] Through this method, the maximum resolution is around 20 to 80 nm. Although the implementation of STED is expensive, it allows for fast data acquisition. Structured illumination microscopy (SIM) is another SRM method which provides high resolution images by utilizing the structured patterns of light illumination across the sample. In SIM, in case of presence of features smaller than the diffraction limit, the applied pattern illumination generates an interference pattern called Moiré fringes.[166] Changing the orientation and position of structured shape, leads to the creation of numerous Moiré fringes which through mathematic deconvolution produce images with 100–150 nm resolution.

Although SIM uses lower illumination power in comparison with other SRM techniques and is adjusted well with conventional microscopy fluorophores and fluorescent proteins, its resolution is lower, especially for virus study. Using the capability of fluorescent dyes to stochastically blink, single molecule localization microscopy (SMLM) was proposed.[167] In this method, the fluorophores which are in the "on" state are controlled precisely to image only a sparse subset at any time. So, the images obtained from individual fluorophores are separated spatially on wide-field detectors. Through accurate localization of the emitted signals from single molecules using Gaussian fit, the reconstruction of images with a 10–20 nm resolution would be possible. Expansion microscopy (ExM) is the latest developed SRM technique to enable high resolution visualization of tissues and preserved cells in conventional diffraction-limited microscopes by the isotropic physical expansion of the specimens in a hydrogel. It is worth mentioning that all of the SRM techniques need the specific and efficient labeling of virions with bright and photostable probes. The main challenge in labeling is to avoid perturbating the functionality of viruses. Several techniques for fluorescently labeling virus particles have been employed, including ipophilic dyes and intercalating, fluorescent proteins and covalent labeling, fluorescent NPs like quantum dots (QDs) and metal NPs, as well as recently proposed methods which exploit fluorescent labeled DNA or RNA probes to hybridize the viral genome,[168,169]MIPS, or a near-instantaneous cation-mediated labeling.[170] In the case of poxvirus and MPXV studies, Jasperse et al. have[171] used light and fluorescence microscopy to study their proposed efficient purification using parental inducer constraint platform for rapid generation of recombinant vaccinia viruses. Furthermore, Johnston et al. have[172] employed fluorescence microscopy to investigate the prophylactic and therapeutic potential of interferon-b (IFN-b) for use against MPXV (**Figure 11**). They show a significant decrease of GFP expression in the presence of IFN-β compared to untreated controls for IFN-β pre-treated, MPXV-GFP-tdTR infected cells. Their results demonstrated the successful inhibition of MPXV using human IFN- β and proposed that IFN- β could be utilized as a safe therapeutic for human MPXV disease.

6.3. Spectroscopy Techniques

The major spectroscopy techniques used to characterize the properties of virus particles are mass spectrometry, nucleic magnetic resonance spectroscopy, vibrational spectroscopy especially Raman spectroscopy, FTIR, and ultraviolet spectroscopy. Moreover, UV irradiation is used to damage the virus and inhibits its infection. In this section, we briefly review the spectroscopy techniques and their application for poxvirus family, especially monkeypox, also see **Table 5** for advantages and limitations of the specific techniques.

6.3.1. Mass Spectrometry

Mass spectrometry (MS) is a method utilized to explore the chemical composition of substances by measurement of their molecular masses. In MS measurements, through vaporizing and ionizing the molecules of interest, the mass-to-charge (m/z) ratios of molecular ions are calculated. MS analysis consists of four steps including ionization, acceleration, deflection, and detection.[179] In the ionization step, by eliminating one or more electrons, the atom or molecule becomes a positive ion. In the second step, the generated ions are accelerated to have the same kinetic energy. Then, using magnetic field, the ions are deflected depending on their masses. There is an inverse relation between the mass of ions and deflection. Finally, the beam of ions that passed through the system is electrically detected. The information about the original ions molecular structure can be revealed by measuring the fragment masses when the gas-phase ions are broken into characteristic fragments. Recently, several mass spectrometry methods have been used to extract the components of viral proteins.[180] Combining these techniques with structural analysis would reveal underlying virus–host interaction dynamics. For the case of pox viruses, mass spectroscopy of the intracellular and extracellular virion of pox viruses resulted in the identification of 164 viral proteins, from both MPXV and VACV, as being virion-associated.[173] Furthermore, although there is a significant overlap between the VACV and MPXV proteomes, MS measurements explored eight unique MPXV-specific proteins consisting of

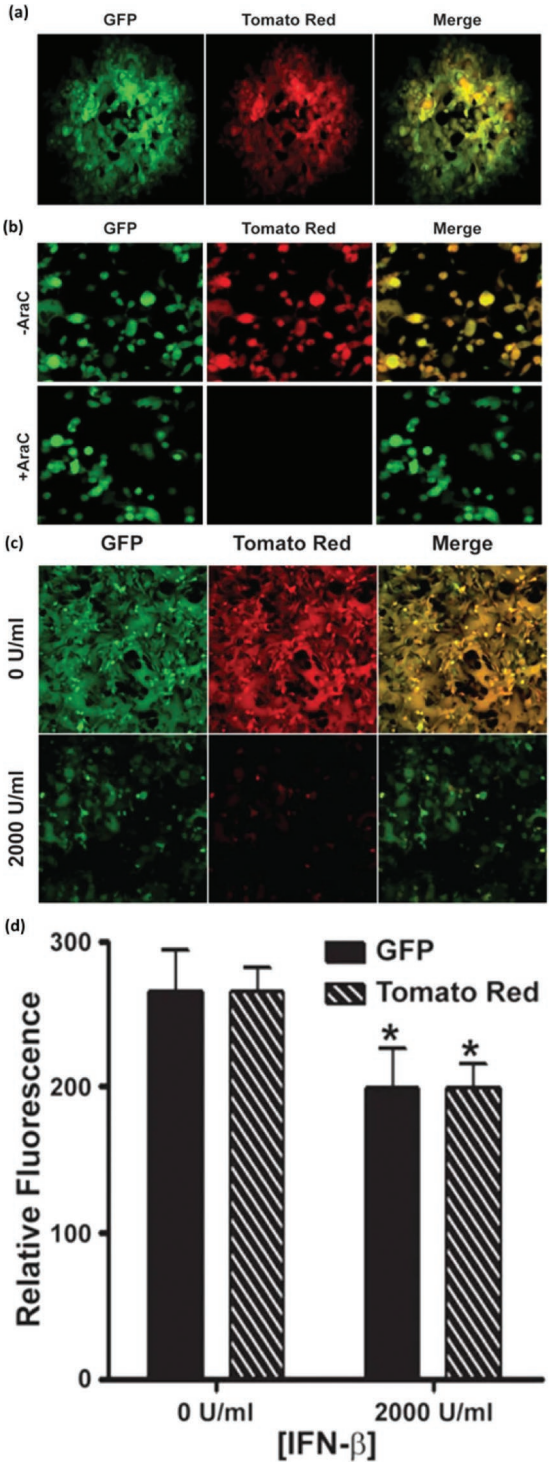

Figure 11. Characterization using flourescence microscopy: a–c) microscopy image of Vero-E6 cells infected with MPXV, GFP, tdTR; d) relative fluorescence extracted from (a–c). Reproduced under terms of the CC-BY license.[172] Copyright 2012, American Society for Microbiology.

MPXV ORFs 002, 003, 010, and 165 which are fragmented in VACV.[181] The identified MPXV ORF 003 encodes an ankyrin (ANK)-containing protein (MPXV-003) that can interact with cellular NF-κB1 and Skp1 and has the ability to inhibit NF-κB signaling activity in human cells via stabilizing NF-κB1 and preventing its degradation.[173,181–183]

6.3.2. Nucleic Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is an advanced spectroscopic technique for virus characterization and determination of its molecular structure at atomic level.[184] The fundamental mechanism of NMR spectroscopy is based on the absorption of radio frequency radiation by atomic nuclei in the presence of an external magnetic field. In virus applications, the multidimensional magnetic field is imposed to the highly purified complexes in solution which excite the particular magnetic resonance of active nuclei in their environment. The chemical shift at which a nucleus resonates relative to a standard is a measure of nucleus-electron-cloud interactions. Generally, the position and number of chemical shifts are indicative of the structure of a molecule and the separations between atoms which allow the 3D reconstruction of the molecule. The major challenge in NMR is the required time to collect and analyze the magnetic resonances and chemical shifts.[185] The size of macromolecule determines the resolution of NMR which is about 100 kDa.[186] The major benefit of NMR is its capability to characterize naturally disordered proteins which is not possible to be performed by X-ray crystallography. Using NMR, the structures of isolated domains of viral proteins such as capsid protein can be revealed. Furthermore, NMR can identify the conformational variations in nucleic acids and viral proteins. NMR can be used as a complementary method with XRD and through integration with electron microscopy can identify pseudoatomic models of entire virus capsids. There are several NMR techniques including the deuteron NMR (H NMR),[187] solid-state NMR (ss-NMR) [101], ^{13}C cross-polarization magic angle spinning NMR (^{13}C CP/MAS NMR),[188] and proton high-resolution magic angle spinning NMR (HR-MAS NMR).[174] Joint use of solid-state and liquid NMR can determine the conformational variations in intact viral capsids on insertion in host membranes. For MPXV, the acquired NMR structures for synthetic peptides with sequences show that one of the "zinc knuckles" of monkeypox possess a conformational transition with an additional C-terminal β-hairpin.[174] This finding suggests packaging of the different genomes may be facilitated due to the dissimilar structural features of zinc knuckles in extensive retroviruses. The poxviruses possess antiapoptotic proteins to confront the defense mechanism of the host cell. NMR results for the VACV protein K7 when explored adopt an α-helical fold of Bcl-2 family of apoptosis regulators, even owing a disassociated amino acid sequence.[189] VACV protein K7 configures complex with the dead-box RNA helicase DDX3 which causes suppression of DDX3-mediated IFN-β promoter induction.[189]

6.3.3. Vibrational Spectroscopy

Vibrational spectroscopy includes two major techniques, infrared and Raman, and their own varieties, which measure the transitions among quantified vibrational states of solids or

Table 5. Spectroscopy techniques and their application for MPXV study.

Spectroscopy technique	Application	Advantages	Limitations	Reference
Mass spectroscopy (MS)	Identification of viral proteins.	No need to exponential amplification of the analyte, so eliminates a potential source of false positives. Identifying the presence of unknown components in a sample.	Difficulty of capture sufficient amount of target material for analysis. High mass-to-charge ratio (m/z) of the virus capsid ions. Direct detection of viruses needs ultrasensitive techniques and expensive equipment.	[173]
Nuclear magnetic resonance (NMR)	Determination of structure/ conformational variations in nucleic acids and viral proteins.	Describe conformational preferences of natively unfolded viral proteins. Simple, fast, and efficient data acquisition and analysis.	Isolation of appropriate virus-like or capsid-like particles. Intrinsic insensitivity.	[174]
Infrared spectroscopy (such as FTIR)	Identification of chemical composition of virus.	High signal-to-noise ratio. Reduced dispersion. Non-destructive high spatial resolution. Straight-forward sample preparation.	Spectral shifts due to CO_2 interference and sample thickness. Possible overlapping of spectral bands between the virus and the host cell.	[175]
Raman spectroscopy/Surface enhanced Raman spectroscopy (SERS)	Whole virus-cell interaction with several small molecules such as drugs. Detection of virus replication and its adhesion to various surfaces.	Sensitive and fast detection of various molecules. Accurate quantification process. Identification and chemical characterization of the analyte.	Low specificity. Requires pretreatment of sample. Needs adequate size of virus.	[176]
Tip-enhanced spectroscopy (TERS)	Virus attachment to the host.	High resolution topography characterization. Identify chemical or molecular information beyond the optical diffraction limitation of sample. Label-free technique.	The enhancement mechanism is not fully known. Tip quality. Possibility of optical path distortion. Lack of standardized methods for consistent and comparable results.	[177]
UV–visible spectroscopy	Determination of concentration of various chemical components of virus	Non-destructive, easy to use and rapid. Minimal data processing and analysis. Inexpensive.	Stray light. Light scattering. Interference from multiple absorbing species. Geometrical considerations.	[178]

molecules.[175] The principal mechanism of vibrational spectroscopy is based on the scattering or absorption of irradiated radiation and collecting the spectrum of spectroscopic responses. The acquired spectrum contains contributions from any molecular bond and is considered as the signature to characterize and identify the variations attributed with physical or chemical processes in materials of interest. In infrared techniques, the transition energy is generally in mid infrared region of collected spectrum (4000–400 cm^{-1}) where the fundamental vibrations are detected. These transitions can be directly measured with (Fourier transform) FTIR absorption spectroscopy or through probing overtones and combinations modes in near infrared (14000–4000 cm^{-1}) region.[190] The basis of Raman spectroscopy is the inelastic scattering of light coupled with vibrational modes of specimen. As water is weaker in Raman spectrum than IR spectrum, Raman spectroscopy has wider applications in the analysis of cells and probing the cellular response to nanoparticles. Moreover, due to the simple structures of viruses as well as separate resolvable wave numbers of Raman bands of protein components and viral nucleic, Raman spectroscopy can be an effective tool in virology.[190] Furthermore, by integration of high spatial resolution of AFM and chemical specificity of Raman spectroscopy, tip enhanced Raman spectroscopy (TERS) is developed which provides the opportunity of chemical imaging at the nanoscale.[177] Surface Enhanced Raman scattering (SERS) effect via active TERS plasmonic probes could enhance the sensitivity similar to how field enhancement takes place in surface plasmon resonances.

Already vibrational spectroscopy especially, Raman spectroscopy, SERS, TERS, and FTIR have been employed to study the physio/chemical compositions of viruses, monitoring infection response, attachment and replication of viruses as well as detection of viruses.[190] Alexander[176] developed a method for the fast discrimination of poxviridae viruses. In his method, whole poxviruses deposited on SERS-active substrates were irradiated with NIR light (λ = 785 nm). His method can be used to classify the unknown Parapoxvirus using their SERS spectral signatures.

6.3.4. Ultraviolet Spectroscopy

The basis of UV–vis spectroscopy is based on the ultraviolet light or visible light adsorption by chemical compounds, which leads to the generation of distinct spectra. The adsorption of ultraviolet radiation causes electron excitation in a substance. This leads to the transition of electrons from a lower energy state to a higher energy state. The difference between these energies is proportional to the amount of ultraviolet radiation or visible radiation absorbed by the substance. UV irradiation and spectroscopy can not only be used to characterize viruses through determination of protein concentration but also be used to treat localized infections.[191] The direct adsorption of UV-C photon by capsid proteins and/or nucleic acids leads to production of the photoproduct that damage the genetic materials in nucleus or nucleic acids in viruses and cause the

virus to become inactive.[191] The damage to DNA and RNA often occurs due to the dimerization of pyrimidine molecules. Hooper et al.[178] have employed UV to determine the concentrations of partially purified A33R, L1R, A27L, and B5R proteins with 280 nm UV absorbance by extinction coefficients of 0.87, 1.52, 8.27, and 0.82 mg^{-1} mL cm^{-1} calculated from the amino acid sequences of the mentioned proteins. They demonstrated DNA vaccine containing four vaccinia virus genes (A27L, L1R, B5R, and A33R) could protect rhesus macaques from severe disease after an encounter with MPXV. This study was the first successful demonstration which used a subunit vaccine approach for immunization of smallpox-monkeypox disease.

7. Conclusions

We discuss several examples here that allow us to understand the state-of-the-art in nanotechnology, which can aid in rapid diagnosis and effective community level surveillance of the MPXV. The fact that the spread of MPXV has gone unnoticed means that we need to prepare advanced diagnostic technology that will prevent such a silent spread of the virus. It is non-trivial to develop a new technology in a quick time that is not susceptible to ethical issues and challenges in technology adoption. However, the promising news is that the technology transfer investments from private and public sector are increasing very fast nowadays. Furthermore, the government regulatory policies in several countries are becoming more business friendly with the researchers/academics. This is the reason we see that there are many companies that have already come up with lateral flow tests for MPXV detection. Most of these available technologies are customized for the specific strain of the MPXV and are based on re-purposing of already well-established sensing technologies such as the lateral flow assay devices. There are well-known limitations of such fast developed technology from the perspective of sensitivity and selectivity. This is where we believe that more efforts in basic sciences and engineering are required to further invent materials that are inherently stable and discover advanced nanotechnology solutions to provide reliability in the management of viral strains that have gone undetected in our community.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

atomic force microscopy, biosensors, monkeypox, nanomaterials, viruses

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