Journal of Nanobiotechnology

REVIEW

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Advanced strategies for the diagnosis, treatment, and vaccination of monkeypox

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Abstract

Monkeypox (Mpox), a zoonotic disease caused by the mpox virus, has been endemic mainly in West and Central Africa for a long time, with only a few cases in other parts of the world. However, recent multi-country outbreaks in non-endemic areas have escalated Mpox into a significant global public health concern. The rapid transmission highlights the urgent need for robust strategies governing diagnosis, treatment, and prevention. In this review, we synthesized advanced approaches addressing the Mpox challenge, focusing on the application of emerging technologies. We examined the integration of nanotechnology, nucleic acid amplification techniques (NAATs), gene editing technology and sequencing technology in Mpox prevention and control. These platforms offered promising solutions to critical limitations in current Mpox control, including high detection costs, poor drug specificity, and suboptimal vaccine efficacy. Finally, we outlined the outlook and potential development trajectories for sustained Mpox prevention and control.

Keywords Monkeypox, Diagnosis, Treatment, Vaccines, Nanotechnology

Introduction

Monkeypox (Mpox) is a zoonotic disease caused by the mpox virus (MPXV),[1] a double-stranded DNA virus belonging to the Orthopoxvirus genus within the Poxviridae family. The first human case of Mpox was identified in the Democratic Republic of the Congo in 1970, initiating limited localized transmission.[2, 3] For decades, Mpox remained predominantly endemic to parts of West and Central Africa. However, in early May 2022, there was a global outbreak of Mpox. This sustained outbreak, reporting over 10,000 cases across more than 50 countries/areas, posed a substantial global public health threat.[4] Consequently, the World Health Organization (WHO) declared the Mpox outbreak a Public Health Emergency of International Concern (PHEIC) in July 2022 [5]. By August 2024, MPXV had reportedly infected more than 100,000 individuals worldwide,[6] with a mortality rate varying between 3.6% and 10.6% in different contexts [3, 7, 8]. Compounding this challenge, the Mpox epidemic has recently resurged. The WHO has, for the second time in two years, designated MPXV as a global public health emergency,[9] underscoring its escalating threat. Alarmingly, this current resurgence exhibits an even higher case fatality rate, exceeding 10% at its peak, thereby imposing more severe consequences on the international community. Therefore, new strategies are urgently needed to control Mpox.

Currently, polymerase chain reaction (PCR) remains a primary method for Mpox detection, valued for its high accuracy and sensitivity. However, Conventional PCR

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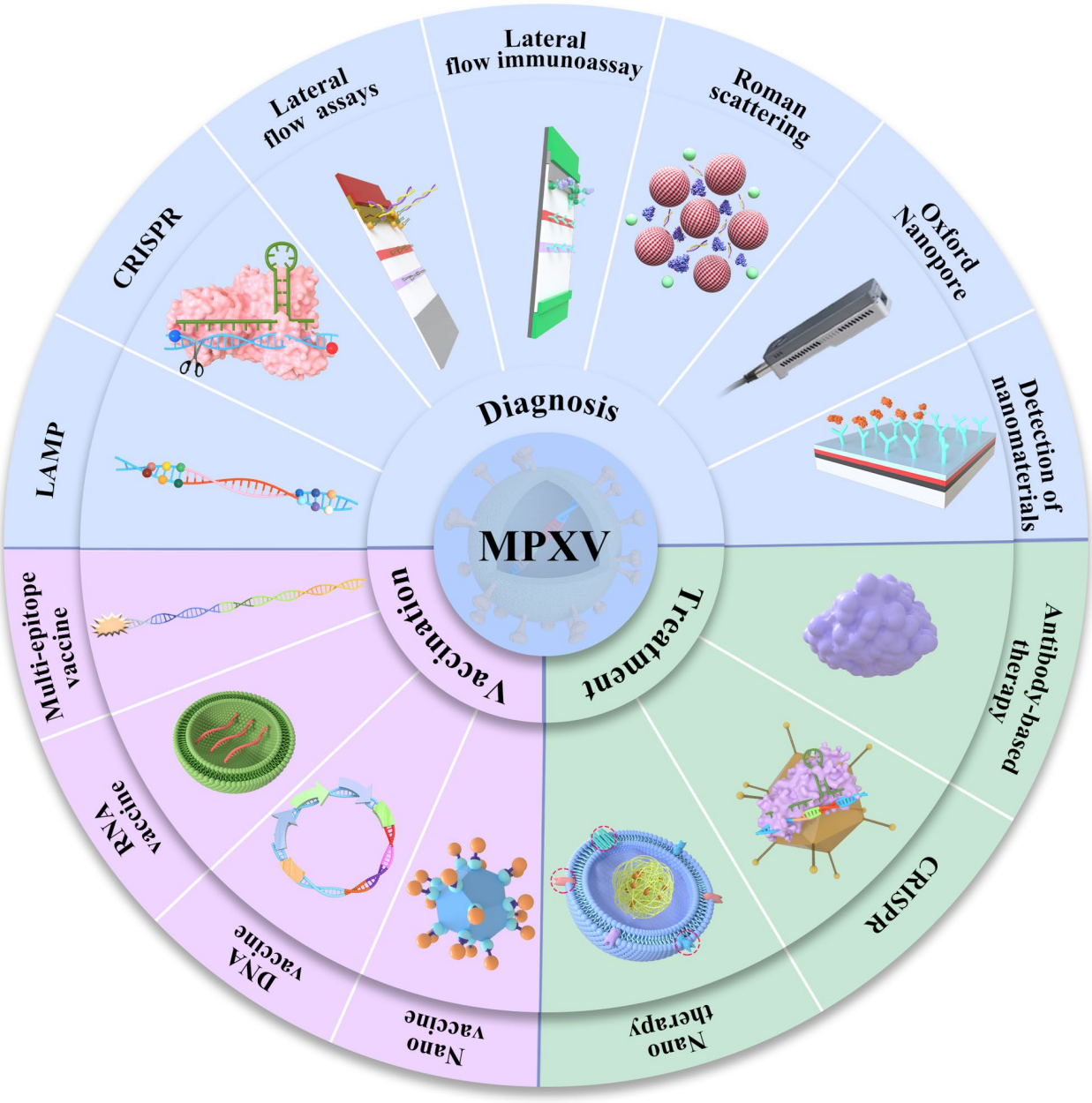
Graphical Abstract



assays are constrained by long run times, high costs and operational complexity, rendering them suboptimal for deployment in point-of-care testing (POCT) [10, 11]. Consequently, emerging diagnostic platforms—including Nucleic acid amplification techniques (NAATs), gene editing technology, sequencing technology, immunochromatography technology and nanotechnology are being actively explored. These alternatives offer advantages such as simple operation, rapid detection, low costs. Their successful application in oncology and virology suggests a strong potential for adaptation to MPXV [12–15]. Beyond diagnostics, significant gaps persist in therapeutics. Current antiviral options, such as cidofovir, vaccinia immune globulin (VIG), and tecovirimat (ST-246) are not specifically designed for MPXV and demonstrate controversial or limited clinical efficacy[16, 17]. Nanotechnology presents a promising alternative. Some special nanoparticles can induce pathogen death via mechanisms like photodynamic, photothermal, and so on [18]. Compared to conventional antiviral drugs, nanotechnology-based therapeutic strategies offer superior target specificity and a lower propensity for inducing

drug resistance [19]. This feature can be harnessed for the targeted killing of MPXV. Therefore, nanotechnology represents a critical tool for the development of novel, highly specific Mpox treatments.

Finally, vaccination remains the cornerstone of Mpox prevention. Amid the recent outbreaks, the U.S. Food and Drug Administration (FDA) issued an Emergency Use Authorization (EUA) for the smallpox vaccine (e.g., JYNNEOS) available for Mpox prevention, leveraging the cross-protective immunity within the Orthopoxviruses [20, 21]. However, the smallpox vaccine is only 85% effective, more importantly, the number of smallpox vaccines available is insufficient.[22–24] To address this, advanced genetic platforms are being utilized to develop next-generation vaccines [25], including mRNA vaccine, DNA vaccine, and multi-epitope vaccine. These excellent follow-up studies may hold promise for MPXV-specific vaccine development in the future. Furthermore, Nanoparticle-based platforms are being investigated as advanced vaccine delivery systems, capable of enhancing immunogenicity, prolonging antigen release, and reducing side effects [26–29]. In view of this, the development of new technologies for the prevention and control of Mpox has flourished in recent years.

There have been a few reports of reviews on Mpox, and these efforts have focused on treatment and vaccine emergency coverage in the 2022 Mpox outbreak. As new technologies are explored in Mpox, there have been many excellent findings. However, there is no comprehensive summary of the application of new technologies to the detection, treatment and prevention of MPXV in recent years. This review aims to bridge this gap by systematically introducing and summarizing recent cutting-edge strategies for MPXV detection, treatment, and prevention. We will delineate the strengths, limitations, and current developmental stages of these approaches, providing valuable insights derived from the evolving landscape of MPXV control. Ultimately, this review seeks to enhance public's understanding of Mpox and provide evidence-based recommendations for scientifically and rationally formulating the strategy for prevention and control of Mpox outbreaks scheme 1.

Novel diagnostic strategies for Mpox

Detection method based on Mpox nucleic acid

Although MPXV infection has obvious clinical manifestations, it frequently presents diagnostic challenges due to its resemblance to other dermatological conditions. Systemic rash associated with Orthopoxvirus infection is commonly misdiagnosed as varicella, herpes simplex or cowpox virus infection [30]. Given the critical role of timely and accurate Mpox diagnosis in contact tracing and transmission reduction, there is an urgent demand for highly precise and rapid MPXV detection methods [31]. Nucleic acid amplification remains a cornerstone in most molecular diagnostic strategies, significantly enhancing detection sensitivity.

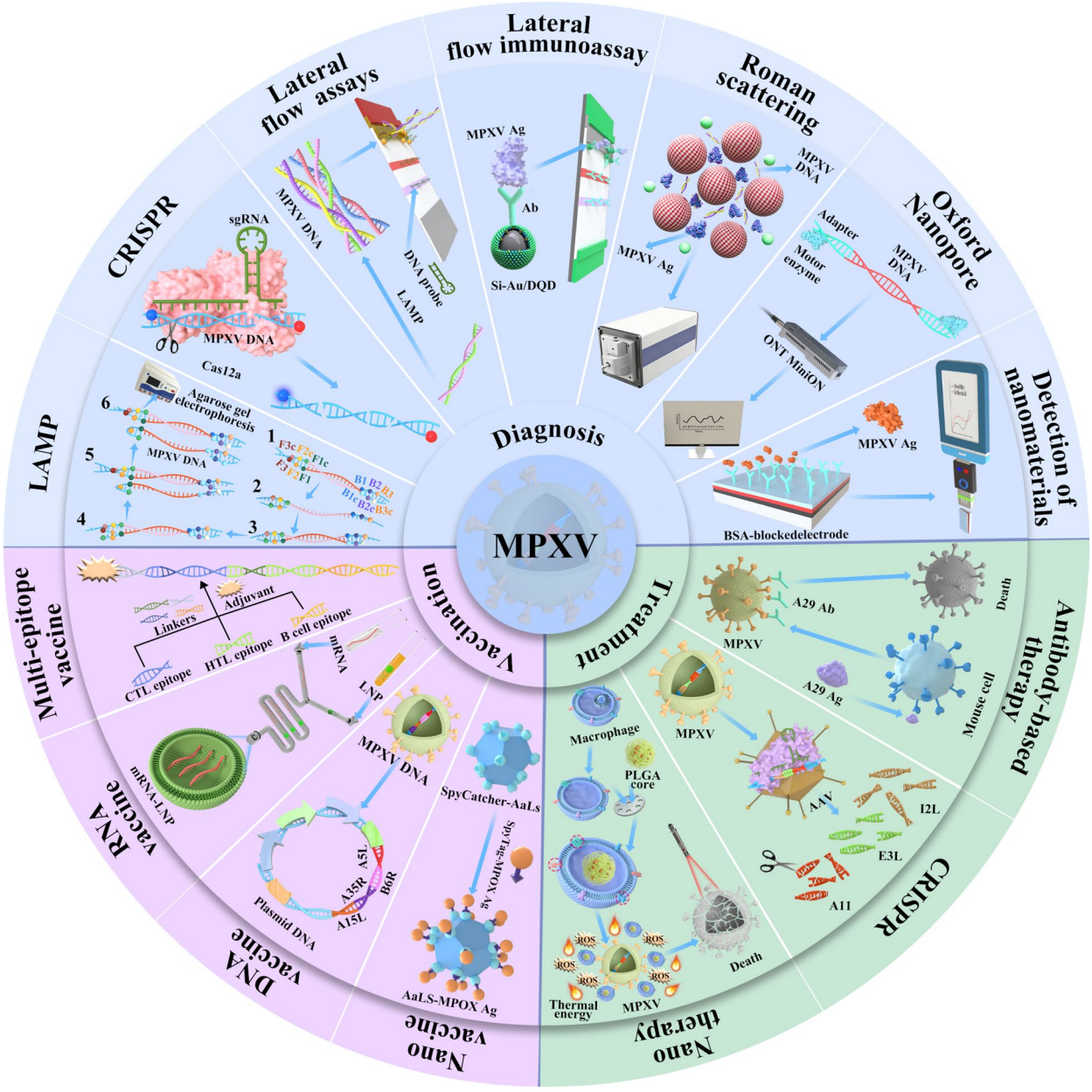
Currently, PCR is a widely adopted method to detect MPXV.[3, 32–34] Historically, PCR assays have been instrumental in differentiating among Orthopoxviruses, including variola virus (VARV), mpox virus (MPXV), cowpox virus (CPXV) and vaccinia virus (VACV) [35–37]. Among the many PCR-based methods, real-time PCR (RT-PCR) is considered to be the gold standard for Mpox nucleic acid detection because of its high sensitivity and specificity [38]. The Mpox Real-time fluorescence quantitative PCR (qPCR) program was announced by the Centers for Disease Control and Prevention (CDC) in June 2022 and improved to eliminate the special characteristics for broader laboratory accessibility [39]. Furthermore, a universal assay for the simultaneous identification of four Orthopoxviruses (VARV, VACV, CPXV, and MPXV) in a single reaction mixture using real-time TaqMan PCR has also been developed by Gavrilova et al [40].

Despite these advancements, PCR-based methods face inherent limitations. The mutable nature of MPXV raises concerns that viral mutations could compromise the effectiveness of traditional PCR primers and probes, thereby impacting diagnostic accuracy. In addition, the PCR method relies on specialized personnel and equipment, and often entails a prolonged turnaround time (TAT), which collectively hinder rapid and accurate detection of the MPXV(Table 1) [10, 41, 42].

Isothermal amplification technique

As alternatives to traditional PCR, new nucleic acid amplification methods such as loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), and recombinase-aid amplification (RAA) offer the advantages of simplified operation, rapid processing, and constant temperature operation [55, 56].

LAMP achieves highly efficient, closed-tube detection. The core of the LAMP methodology involves a strand-displacement DNA polymerase (e.g., Bst enzyme) and a set of four to six specialized primers (Forward Inner Primer, FIP; Backward Inner Primer, BIP; and two outer primers, F3 and B3). These primers are designed to recognize multiple distinct regions on the target sequence. This intricate primer design initiates an auto-cycling, self-priming amplification process that produces amplicons with characteristic stem-loop structures, leading to exponential accumulation of DNA. This mechanism, which proceeds at a single temperature without an initial thermal denaturation step, allows the entire reaction to be completed in a sealed tube. This integrated strategy obviates the need for sophisticated thermal cycling instrumentation and minimizes the risk of carryover



Scheme 1. A graphical overview of new strategies for the diagnosis, treatment and vaccination of monkeypox. The applications of advanced technologies in the diagnosis of monkeypox mainly include loop-mediated isothermal amplification (LAMP) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), lateral flow assay, lateral flow immunoassay, Raman scattering, Oxford Nanopore, nanomaterials, etc. The application of advanced technologies in the treatment of monkeypox mainly includes Antibody-based therapy, CRISPR and Nano therapy, etc. The titer of MPXV DNA in the diseased mice after their treatment was significantly reduced. The application of advanced technologies in monkeypox vaccines mainly includes Nano, DNA, RNA and Multi-epitope vaccines, etc. DNA or RNA vaccines are new vaccine platforms constructed based on the mRNA and DNA of key antigen proteins on the surface of mature virus particles (MV) and enveloped virus particles (EV), respectively. The multi-epitope vaccine is designed by selecting epitopes of highly antigenic cytotoxic T lymphocytes (CTL), helper T lymphocytes (HTL), and B cells (B cell)

(aerosol) contamination. Furthermore, the large amount of DNA produced allows for simple, visual interpretation of results, often via turbidity or colorimetric changes from pre-mixed indicators. These features make LAMP exceptionally well-suited for on-site, rapid, and POCT [44].

However, the primary limitation of LAMP lies in its complex primer design. The requirement for multiple primers not only increases the initial setup cost and design 20complexity but also renders the assay more vulnerable to target sequence variations. When applied to viruses with high mutation rates, a single nucleotide polymorphism (SNP) in any of the multiple primer-binding

Table 1 Advanced Techniques for Mpox Detection

Technology type	Detection target	Detection time	Limit of detection	Equipment requirements	Applicable scene	Major advantage	Major disadvantage	Reference
PCR	Viral DNA	2-4 h	100–1000 copies/μL	PCR amplification apparatus	Diagnosis and early diagnosis	Gold standard	Rely on equipment and professionals	[38, 41]
LAMP	Viral DNA	30 min	2 copies/μL	Thermostatic equipment	Rapid field screening	Simple, fast, and not dependent on technology	Primers are difficult to design	[43, 44]
CRISPR	Viral DNA/RNA	30 min	10 copies/μL	Thermostatic equipment, fluorescence reader	Rapid field screening	Double target detection, portability potential	Rely on professionals	[43, 45]
LFA	Viral proteins/ nucleic acids	15 min	100 ng/mL /20 copies/μL	No equipment required	Convenient detection	Instant detection, simple operation	Low sensitivity and low light signal	[46–48]
Oxford Nanopore Technologies	Viral whole genome	Several hours		Portable sequencer	Strain tracing	Real-time sequencing, portable	Rely on equipment	[49, 50]
Raman scattering	Viral proteins/ nucleic acids	10-60 min	5 ng/mL/ 0.625 μM	Raman spectrometer	High sensitivity and fast detection	No labeling required	SERS signal interference	[51, 52]
Luminescent carbon quantum dots	Viral proteins	15-60 min	0.61 μg/mL	biosensor	Portable fluorescence detection	Customizable probe	Stability is to be verified	[53, 54]

Abbreviations: LAMP, Loop-mediated isothermal amplification; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats.

sites can severely compromise or completely inhibit amplification efficiency.

Isothermal amplification has been proved to be an alternative to RT-PCR. LAMP is a simple, rapid and technology-independent detection method. Since Nozomi et al. was proposed [57], LAMP-based diagnosis has been developed to detect pathogens such as viruses [58, 59], bacteria[60] and fungi [61]. Gary et al. designed primers for the inner and surrounding regions of MPXV N4R gene and established an isothermal LAMP for specific detection of Mpox (Fig. 1A) [62]. The detection limit of the method is 2×10^0 DNA copies and resembles the qPCR method used for MPXV detection. Importantly, the test provides visual results to the eye-visible results in less than 30 min (Fig. 1B). However, LAMP-based detection methods require the design of specific multiple primers, which increases the cost and is difficult to combat viral mutagenicity [44].

Compared with LAMP, RPA and RAA technologies offer simplified primer design, typically requiring only a single pair of primers to achieve efficient amplification, while maintaining high sensitivity and isothermal reaction conditions.[55, 63] These enzymatic DNA amplification methods enable rapid amplification within 15 min at temperatures between 37 °C and 42 °C. For example, Ahmed et al. developed an RPA assay targeting the MPXV G2R gene, enabling rapid detection of MPXV branches in Central and West Africa [64]. The RPA assay, conducted at 42 °C, delivers results in 3 to 10 min with a detection limit as low as 16 DNA molecules/μL, surpassing most LAMP systems in terms of reaction speed and sensitivity.

To further enhance specificity and streamline result interpretation, researchers have explored integrating RPA and RAA with complementary technologies. Nicolas et al. established and validated three isothermal amplification methods incorporating recombinant enzymes: RPA combined with CRISPR-Cas12a (RPA-Cas12a), real-time RAA or RPA combined with lateral flow assays for rapid detection of MPXV isolates. lateral streamer combined with real-time RAA and RPA for rapid detection of MPXV isolates [65]. This method can detect a minimum of 10^0 copies of DNA, outperforming conventional PCR techniques (Fig. 1C, D). Moreover, they address the limitations of traditional diagnostics by enabling visual result interpretation within 20–30 min, eliminating the need for time-consuming processes and specialized equipment. This integrated approach not only surpasses PCR in sensitivity but also highlights the potential of combining isothermal amplification with advanced detection technologies for robust, field-applicable mpox diagnostics.

To enhance technology integration and enable field-deployable diagnostics, Li et al. developed an innovative solution combining rapid nucleic acid extraction via paper-based test strips with LAMP performed in a closed, multi-channel microfluidic chip. [66] This platform demonstrated high sensitivity and specificity, with its portability and low cost making it particularly suitable for resource-limited settings [67]. However, challenges remain in improving analysis speed and controlling

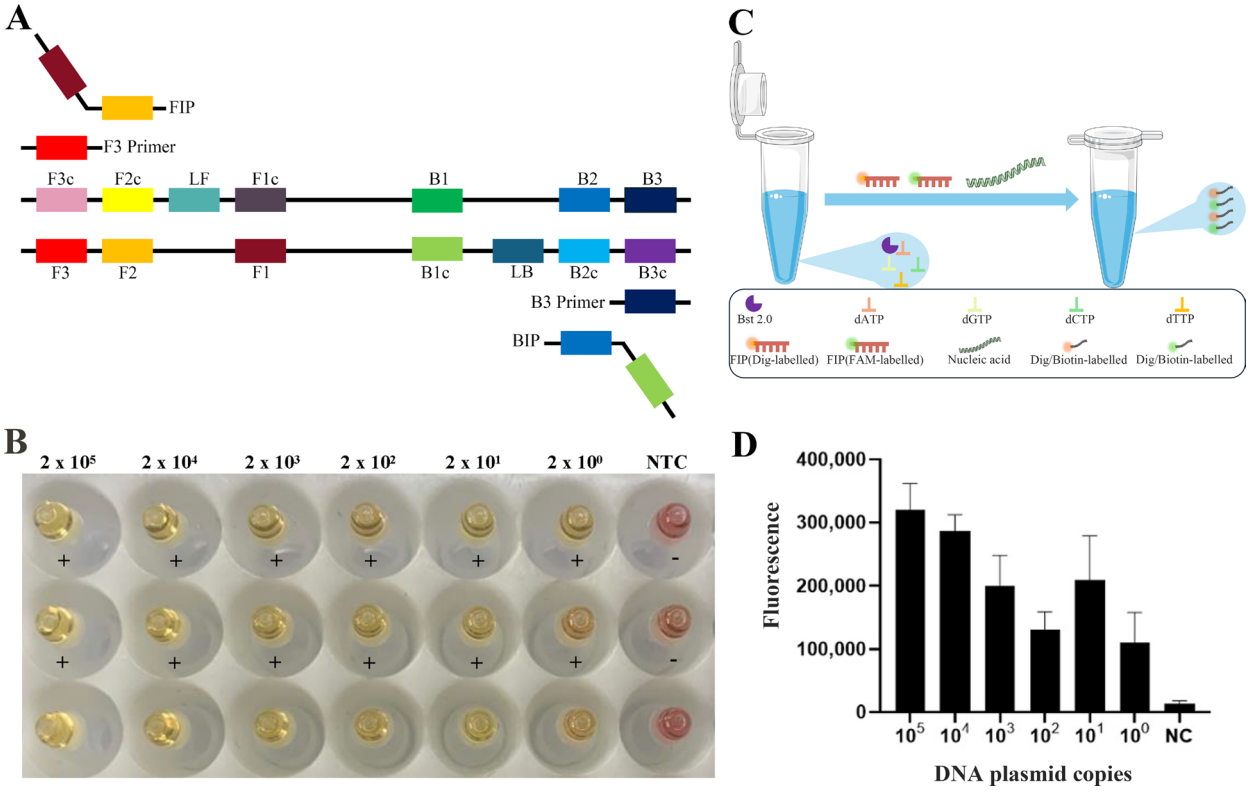


Fig. 1 Application of isothermal amplification in MPXV detection. **A**) Schematic representation of the location of primer recognition sites around the N4R gene, the gene is duplicated in the left and right inverted terminal repeats (ITR) of the Mpox genome. **B**) Through a change of color from pink to yellow, the sensitivity of the visual LAMP assay was assessed. **C**) Fluorescence values of real-time RPA after 50 cycles. Reproduced with permission [69]. Copyright 2023, Talanta. **D**) Fluorescence values of RPA-Cas12a after 50 cycles. Reproduced with permission. [65] Copyright 2022, Viruses

contamination risks. Addressing these limitations, Huang et al. designed a multifunctional microfluidic chip that integrates nucleic acid extraction, amplification, and detection. This system employs ultrasonic-assisted mixing and lysis to accelerate processing, optimizes microvalve and flow channel designs for rapid, automated fluid control, and reduces processing times from minutes to seconds. To mitigate contamination, the chip utilizes closed-tube detection, with all processes completed within a sealed environment, achieving a "sample-in, result-out" workflow. Reagents are pre-sealed in the chip's storage chambers and released via pressure, eliminating the need for frequent cap opening or pipetting, thereby minimizing contamination risks. These studies highlight the transformative potential of combining microfluidic technology with isothermal amplification to achieve automated, contamination-free, and portable Mpox diagnostics, underscoring technology integration as a critical pathway for field applications.

Isothermal amplification techniques offer significant advantages over traditional PCR in terms of speed, operational simplicity, and equipment requirements, making them ideally suited for rapid field screening and POCT. However, they are not without limitations. These include a higher risk of false-positive results due to aerosol contamination during tube opening, difficulties in designing multiple specific primers and generally inferior quantitative capability compared to qPCR [68]. Currently, while LAMP and RPA/RAA platforms are well-established, specific assays for MPXV detection are primarily in the late-stage proof-of-concept or preclinical validation phase, with some integrated prototypes being developed for future clinical application [43, 44].

CRISPR

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system, a core component of microbial adaptive immunity, utilizes CRISPR-associated (Cas) endonucleases (like Cas12a and Cas13a) to recognize and cleave foreign nucleic acids with high sequence specificity [70]. This high-fidelity mechanism has positioned CRISPR technology as a revolutionary tool for nucleic acid detection, offering exceptional specificity, accuracy, and operational simplicity [71]. It is widely regarded as a next-generation diagnostic platform [72]. However, the native CRISPR-Cas system lacks the analytical sensitivity required to detect the low concentrations of target nucleic acids typical in clinical samples (Fig. 2A),

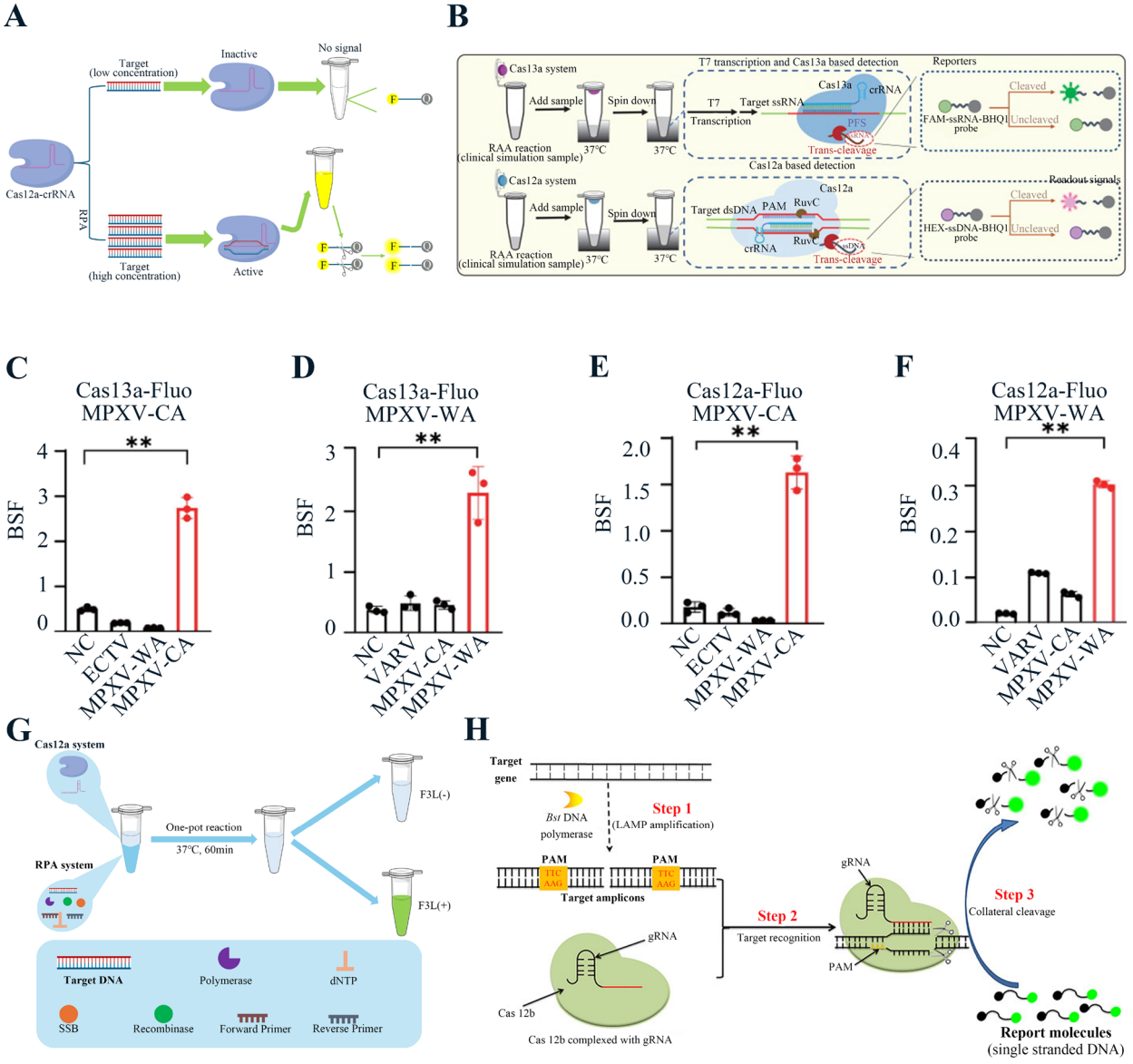


Fig. 2 Application of CRISPR system in MPXV detection. **A**) The Cas12a effector remains inactive in the absence or at low concentrations of target DNA and can not cleave single-stranded DNA (ssDNA) reporters. Target amplification results in activation of the Cas12a effector by combining recombinase polymerase amplification (RPA). **B**) Diagram of the OP-RAA-Cas12a or OP-RAA-Cas13a one-pot single-target detection system. **C**-**F**) Specificity analysis for Cas12a-Fluo and Cas13a-Fluo detection assays. Reproduced with permission [43]. Copyright 2023, Science Bulletin. **G**) The diagram showing CRISPR and RPA reactions divided by a dynamic interface between the two phases. Reproduced with permission [71]. Copyright 2022, Microbiology Spectrum. H) Schematic diagram of the LAMP-CRISPR/Cas12b assay used to detect MPXV. Reproduced with permission. [78] Copyright 2023, Microbiology Spectrum

Consequently, coupling CRISPR detection with a preceding nucleic acid amplification step is essential. While traditional PCR faces limitations in rapid, point-of-care settings [41, 42], the advantages of isothermal amplification methods (LAMP, RAA, or RPA) in terms of speed and single-temperature operation make them ideal partners for CRISPR [55]. This integration has created a new, promising class of rapid diagnostic assays [73].

This synergy between isothermal amplification and CRISPR has been effectively demonstrated in various configurations. For instance, Du et al. developed an assay combining RAA with both Cas13a or Cas12a to successfully develop a detection method based on RAA-Cas12a/Cas13a [43], which enables detection of two gene targets of Mpox by incorporating the Cas12a and Cas13a systems in one sample and making the reaction system large-scale (Fig. 2B). In addition, the dual-target detection strategy has a strong specificity (Fig. 2C-F), which can distinguish MPXV-WA and MPXV-CA branches in 10 min after RAA. However, the limitation of this specific assay was its focus on intra-species differentiation rather than broad-spectrum Orthopoxvirus screening.

To further enhance diagnostic specificity and differentiate MPXV from related viruses, other groups have refined this approach. Guo et al. developed a rapid, high-sensitivity MPXV detection system combining RPA with CRISPR-Cas12a [45]. They strategically designed a set of CRISPR-derived RNAs (crRNAs): one group targeted conserved genes (D6R and E9L) for pan-Orthopoxvirus detection, while another group targeted genes specific to variola virus (N3R and N4R) for simultaneous identification. This entire differential detection assay was completed in 30 min at 37 °C, achieving a sensitivity of fewer than 10 viral DNA copies per reaction. Similarly, another RPA-CRISPR method was developed to specifically distinguish MPXV from other orthopoxviruses by targeting a single nucleotide polymorphism (SNP) in the conserved Pola (E9L) gene [74]. Other assays have employed Cas12a and Cas13a to detect the MPXV-specific genes F3L and B6R, respectively [75]. The results from these systems are often visualized using simple lateral flow strips or portable fluorescent readers, enhancing their utility in point-of-care settings [76]. A common limitation of these initial strategies, however, was their reliance on multi-step operations (e.g., opening the tube after amplification to add CRISPR reagents). This introduces operational complexity and, more critically, a significant risk of carry-over contamination, challenging their broad applicability.

To directly address the inherent operational complexity and contamination risks in multi-step detection, subsequent research has focused on developing an optimized "one-pot" system [77]. The MPXV RPA-CRISPR one-pot method is a prime example, integrating all amplification and detection reagents into a single, sealed reaction vessel [71]. This approach simplifies the workflow from a two-step analysis to a single step, significantly reducing the risk of contamination. By carefully optimizing reagent compatibility (Fig. 2G), these one-pot systems retain the high specificity, accuracy, and operational convenience of the CRISPR-Cas platform, making them robust candidates for future diagnostics. Beyond Cas12a and Cas13a, other CRISPR-Cas enzymes also present advantageous diagnostic capabilities. For example, Li et al. integrated the CRISPR-Cas12b system with LAMP to develop a novel MPXV nucleic acid assay specifically targeting the A-type Inclusion Body (ATI) gene [78]. In this assay, LAMP facilitates the specialized amplification of target genes containing specific loci (e.g., a PMA locus). Subsequently, the amplified target fragments are captured by a specific guide RNA (gRNA), which forms a complex with CRISPR-Cas12b. Upon recognizing a matching target sequence, the gRNA-CRISPR-Cas12b complex induces the non-specific collateral cleavage of single-stranded DNA reporter molecules, generating a detectable signal (Fig. 2H).

The integration of CRISPR with isothermal amplification thus represents a highly promising next-generation diagnostic platform, characterized by exceptional specificity, rapid turnaround times, and inherent portability. Nevertheless, several key challenges must be addressed. for widespread clinical adoption. These include the financial cost and design optimization of crRNAs, the inherent complexities of multi-step assay procedures which elevate the risk of contamination, and the persistent requirement for a pre-amplification step to achieve clinically relevant sensitivity thresholds [43, 45]. Currently, nearly all reported CRISPR-based assays for MPXV remain in the proof-of-concept or early preclinical development stage [79]. While analogous CRISPR diagnostics have transitioned into clinical use for other pathogens (e.g., SARS-CoV-2) [80], MPXV-specific CRISPR platforms are still under active development and await broader deployment in clinical settings.

Lateral flow assay

The lateral flow assay (LFA) is a widely recognized, portable, and rapid diagnostic technique capable of quickly analyzing spiked samples.[81] Its operational principle relies on capillary action, which drives a processed sample along a porous polymer strip through various reaction zones. Within this strip, specific biorecognition molecules (e.g., antibodies, oligonucleotides) bind to the target analyte and are conjugated to reporter particles, typically colloidal gold or latex microspheres, which provide a colored or fluorescent signal. As the sample migrates along the test strip, the analyte-reporter conjugate sequentially encounters pre-immobilized capture lines (test line) and a control line. Specific immune recognition or hybridization events at these lines lead to the aggregation of reporter particles, resulting in a visually interpretable signal. For automated and quantitative analysis, a portable biosensor can precisely capture the reflected light or fluorescence signals from the test and control lines via an integrated optical module. These biochemical signals are then amplified, converted from analog to digital, and algorithmically analyzed by a microprocessor, ultimately displaying quantitative detection results. This integration achieves automation and precision in rapid diagnostics.

Nanoparticle-based lateral flow biosensors are particularly attractive due to their ease of use, rapid turnaround, low cost, and accessibility [47]. This technology has been extensively applied in disease screening and pathogen detection [82, 83]. With the global emergence of Mpox, the demand for fast, accurate, and user-friendly diagnostics has surged, positioning nanoparticle-based LFA biosensors as a highly promising tool for MPXV detection. LFA is broadly categorized based on their target analytes: lateral flow immunoassay (LFAI), which relies on immunochromatographic principles to detect

antigens or antibodies, and nucleic acid lateral flow assay (NALFA), which utilizes oligonucleotide hybridization to detect nucleic acids [81]. NALFA, when coupled with nucleic acid amplification (e.g., LAMP, RPA, RAA), significantly lowers the detection limit by exponentially increasing viral nucleic acid targets. This combination of robust amplification and LFA's rapid, visual readout enhances both the specificity and sensitivity of the assay while retaining its inherent convenience and speed. The successful integration of nucleic acid amplification with LFA for the detection of viruses such as SARS-CoV-2 and influenza[84, 85] strongly supports its potential for MPXV diagnostics. A compelling example is the MPXV-LAMP-LFB assay developed by Huang et al., which combines a nanoparticle-based lateral flow biosensor (LFB) with LAMP.[86] In this method, six pairs of LAMP primers were specifically designed for the MPXV ATI gene, and template amplification was performed at 63 °C in only 40 min. The test results, interpreted via the LFB strip, were rapidly and visually discernible within just 2 min. This approach exhibited high specificity, demonstrating no cross-reactivity with other non-MPXV pathogens, thereby enhancing the accuracy of MPXV detection.

Building upon this methodological foundation, Xiao et al. further validated the broad applicability and discriminatory power of this technology. By designing primers for additional target genes (e.g., D14L and ATI), they successfully adapted the MPXV-LAMP-LFB assay for the differentiation of MPXV genetic clades [69]. Critically, the MPXV-LAMP-LFB assay can be conveniently performed using a simple heating block, and results are easily visualized through the biosensor, eliminating the need for specialized laboratory equipment (Fig. 3A, B).

While the LAMP-LFB method offers a favorable balance between speed and specificity, ongoing technological innovations. continue to push the boundaries of sensitivity. Multiple cross-displacement amplification in combination with LFB (MCDA-LFB) has emerged as a particularly sensitive method for diagnosing MPXV infection and differentiating between West and Central African MPXV strains [87]. The MPXV-MCDA-LFB assay demonstrated exceptional sensitivity, capable of detecting target genes with as few as 5 copies of plasmid template and 12.5 copies of pseudo typed viruses per reaction. This effectively addresses the inherent sensitivity limitations of many traditional lateral flow platforms.

To systematically evaluate the performance of various nucleic acid amplification-based LFA technologies, Wu et al. conducted a comparative study [88]. They integrated helicase dependent amplification (HDA) (Fig. 3C) and recombinase polymerase amplification (RPA) (Fig. 3D) with LFA (Fig. 3E), alongside their internally developed qPCR technique, to identify the MPXV-specific conserved fragment F3L (Fig. 3F-H). This research assessed the validity of these methods with the objective of establishing effective MPXV tests for early diagnosis and screening. Importantly, this study moved beyond the development of a single combined detection method, offering a direct comparison of different approaches (HDA-LFA, RPA-LFA, and qPCR-LFA). This provides a clearer understanding of their relative advantages concerning specificity, sensitivity, and practicality in MPXV detection. The researchers also assessed the validity of these methods with the objective of establishing an effective test of MPXV for early diagnosis and screening [88]. This study goes beyond the development of a single combined detection method and directly compares different methodological approaches (HDA, RPA, qPCR combined with LFA), providing a clearer picture for understanding their relative advantages in terms of specificity, sensitivity and practicality in MPXV detection.

Ultimately, LFAs are distinguished by their unparalleled speed, ease of use, low cost, and suitability for decentralized testing, making them ideal for large-scale screening and resource-limited settings. Their primary drawback, however, remains their relatively lower inherent sensitivity compared to laboratory-based nucleic acid amplification methods, which can lead to false negatives, particularly in early infection stages.[46–48] The examples discussed, from the foundational MPXV-LAMP-LFB to the more sensitive MPXV-MCDA-LFB, and the comprehensive comparative study of HDA/RPA/qPCR-LFA, collectively illustrate the research community's strategic approach to mitigate this sensitivity limitation by integrating diverse isothermal amplification techniques. While the underlying LFA technology is mature, MPXV-specific NALFA platforms, which combine amplification with lateral flow detection, are predominantly in the preclinical research and development phase. Current research is actively advancing towards the goal of developing highly sensitive, equipment-free, and rapid Mpox POCT.

Oxford nanopore technologies

Oxford Nanopore Technologies (ONT) offers a revolutionary platform for long-read sequencing methods (LRS), enabling the recovery of full-length RNA molecules—a feature critically important for accurate transcriptome annotation.[89, 90] For Mpox, ONT's capabilities are particularly pertinent given the higher-than-expected frequency of MPXV single-nucleotide polymorphisms (SNPs), which increases the likelihood of emerging variants with altered pathogenicity.[11, 91] Therefore, nanopore sequencing provides an invaluable tool for comprehensively understanding and predicting the evolution of Mpox epidemic. In this context, rapid nanopore genome sequencing methods have facilitated

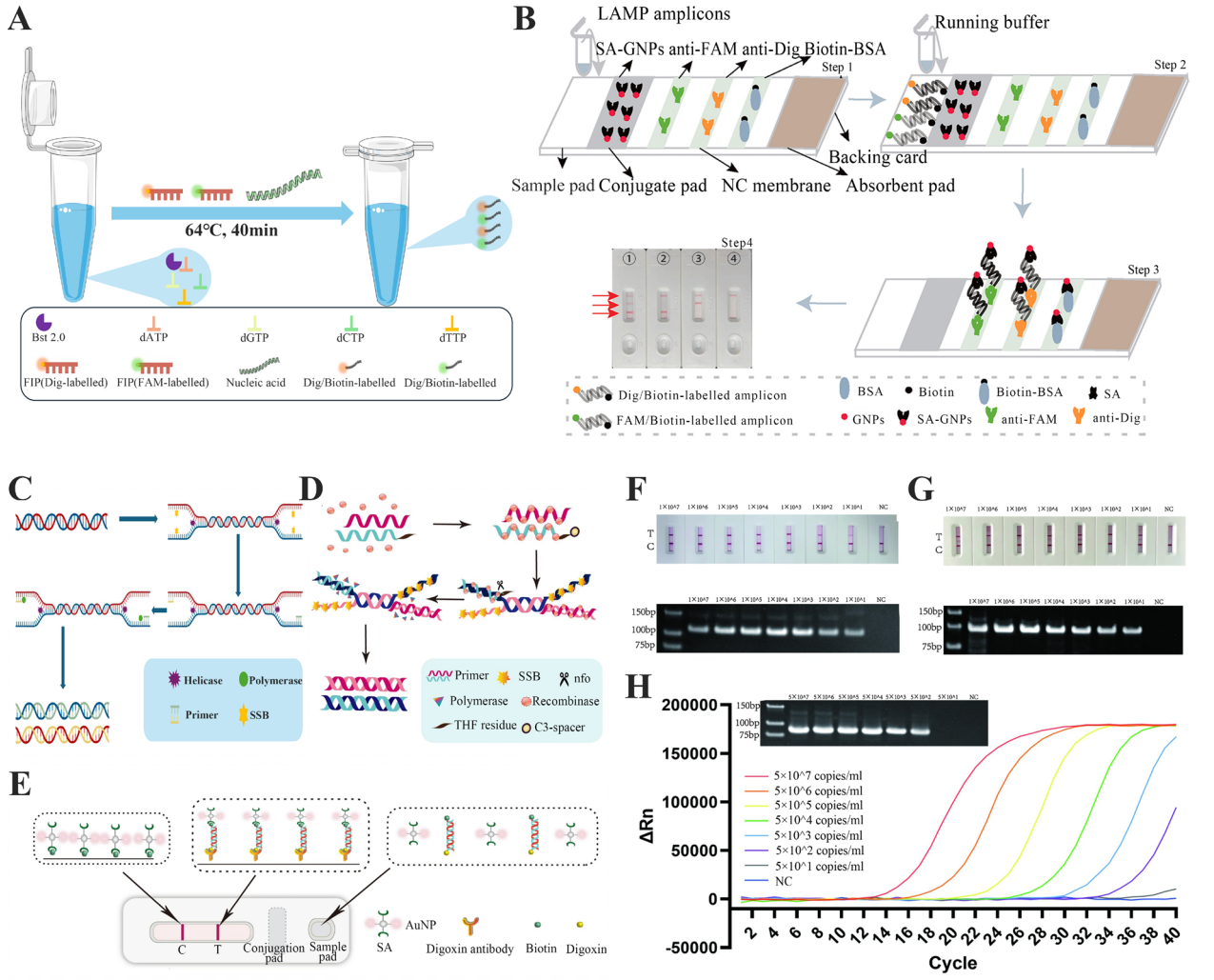


Fig. 3 Application of nucleic acid LFA in MPXV detection. **A**) MPXV-LAMP assay procedure. **B**) LFB Principles and Procedures for MPXV-LAMP Products (Steps 1–3) and Interpretation of MPXV-LAMP-LFB Results (Step 4). Reproduced with permission [69]. Copyright 2023, Talanta. **C**) Basic principle of HDA amplification. **D**) Fundamental principle of RPA amplification: A tetrahydrofuran modification site is introduced into the probe and the 3′ end is modified to prevent extension. E) Fundamental principle of the LFT technique: Digoxin antibody was immobilized on the strip membrane (T-line), and then colloidal gold was immobilized on the strip membrane (T-line). **D**-**H**) Sensitivity testing of qPCR, RPA-LFT, HDA-LFT. Reproduced with permission [88]. Copyright 2023, Virology Journal

the sequencing of multiple complete MPXV genomes, encompassing a streamlined workflow from DNA extraction to phylogenetic analysis.[92, 93] This approach not only simplifies near-real-time MPXV tracking and rapid phylogenetic discovery but also establishes a blueprint for deploying ONT to monitor diverse viruses and anticipate future outbreaks, thereby mitigating the threat posed by the undetected emergence and spread of novel pathogenic variants.

Leveraging ONT, a research team developed Rapid Amplification Nanopore Sequencing (RANS) for Mpox identification [94]. RANS integrates multiplex PCR amplification with direct ONT sequencing by simultaneously incorporating dA tails and 5' phosphonates into PCR products, facilitating their direct ligation to ONT adaptors (Fig. 4A). After rapid sequencing (within minutes), the generated reads are mapped against a reference database to identify the most recent viral strain. This method offers significant advantages in virus identification, being faster, more accurate, and more cost-effective than conventional approaches. Crucially, RANS overcomes the inherent limitations of traditional PCR methods in broadly characterizing Orthopoxviruses [95].Concurrently, ONT MinION long-read sequencing devices have been utilized to sequence the transcriptomes of Mpox and its host cells.[96] De novo cDNA (dcDNA) sequences were obtained from infected cells at six distinct time points, providing a dynamic view of viral transcription. Elucidating the transcriptional profile of MPXV is vital for a deeper understanding of viral biology,

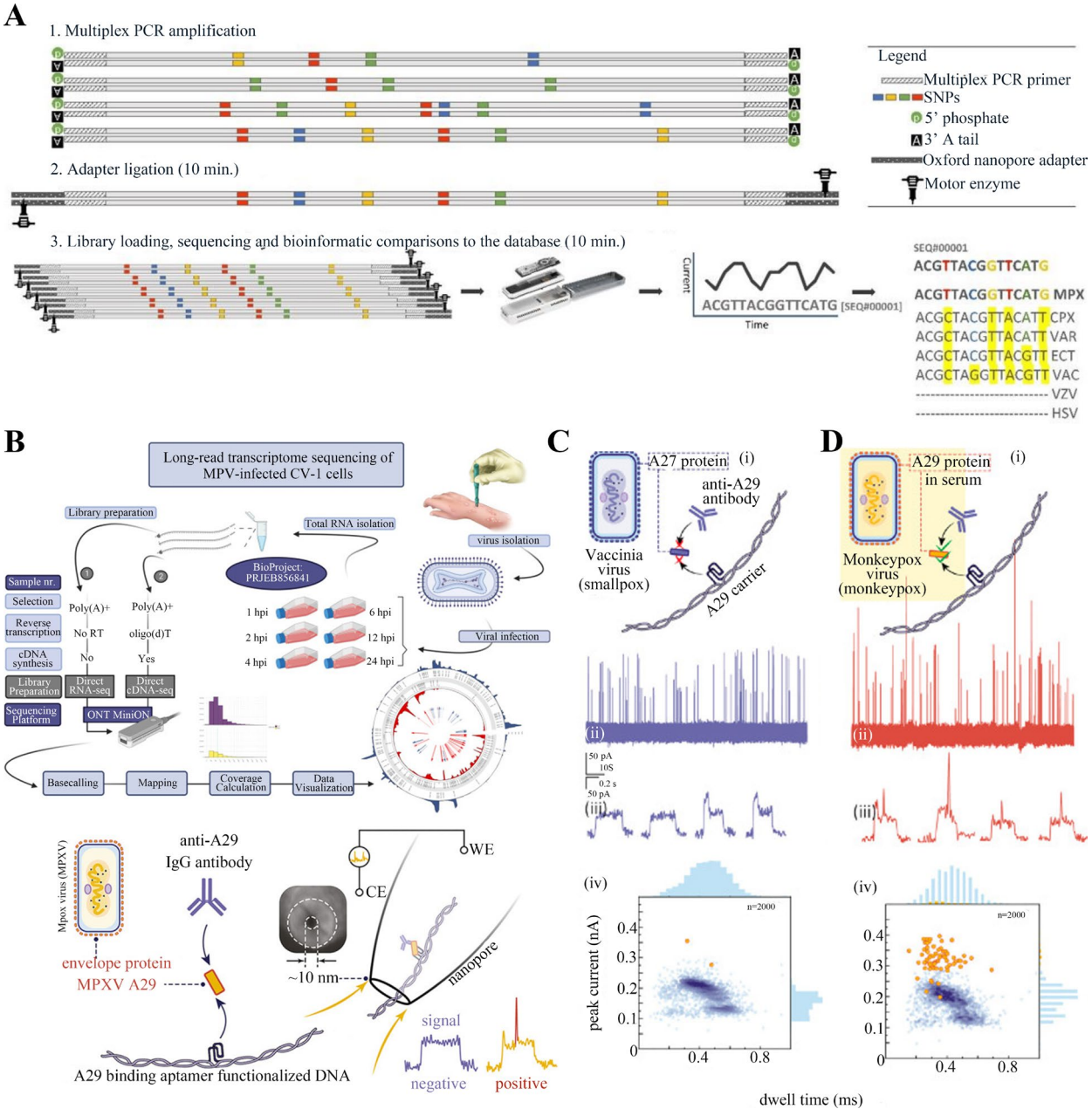


Fig. 4 Application of nanopore technology in MPXV detection. **A**) The Rapid Amplicon Nanopore Sequencing (RANS) method for the diagnosis of MPXV and other pathogens which can form vesicles. Reproduced with permission [94]. Copyright 2022, Viruses. **B**) After the indicated time of infection, the total RNA was separated and sequenced on ONT's Minion platform using a direct cDNA sequencing protocol. Reproduced with permission [96]. Copyright 2023, Scientific Data. **C**) Schematic current–time diagram of the transport of a synthetically labeled molecular probe through a nanopore in the presence of the A29 protein and its antibody. **D**) The specificity of the MPXV A29 protein in human biofluids. (i), representative current–time trajectories (ii), typical events (iii), and residence time and peak current statistics (iv) for DNA molecular probes translocated in the presence of Vaccinia virus A27 protein. **C**-**D**) Reproduced with permission [49]. Copyright 2023, Nano Letter

which can ultimately accelerate the development of effective therapeutic interventions.

To push the boundaries of detection speed, simplicity, and move towards POCT, Guan et al. developed a highly specific and sensitive MPXV detection system called CRISPR-Cas12A-assisted nanopore (SCAN), integrated with RPA [50]. After collecting and preparing swab samples, RPA was performed at 39 °C for 20 min. Subsequent activation of the CRISPR-Cas12a reaction leads to the collateral cleavage of a single-stranded DNA reporter molecule. The final detection is achieved via nanopore reading, where the cleavage events are sensed as changes in electrical current. This innovative strategy combines the specificity of RPA-CRISPR with the sensitivity of

nanopore sensing, poised to open a new frontier for POCT applications. Furthermore, ONT's intrinsic sensing capabilities extend beyond nucleic acids to directly detect protein biomarkers, offering a complete alternative to traditional nucleic acid testing. This approach utilizes nanopores for the selective, single-molecule detection of the mpox virus A29 protein in biological fluids. The method employs customized DNA molecular probes, ingeniously incorporating both an antibody and an aptamer to enhance single-molecule detection of Mpox A29 protein. The aptamer-target and antibody-sandwich structure facilitates efficient target recognition as the protein passes through the nanopores (Fig. 4C) [49]. This protein-level detection completely bypasses the need for nucleic acid amplification, providing a direct pathway for diagnosing active infections. Moreover, it accurately detects the A29 protein at concentrations as low as 11 fM (in the context of vaccinia virus) and precisely distinguishes it from the vaccinia virus A27 protein (which differs by only four amino acids) and varicella zoster virus proteins directly in bodily fluids (Fig. 4D). This exemplifies ONT's potential for rapid, accurate, and direct MPXV detection.

In summary, ONT sequencing offers unparalleled advantages for real-time genomic surveillance, outbreak tracing, and identifying new viral variants, primarily due to its long-read capability and portability [92, 93]. However, inherent limitations include a relatively high raw read error rate compared to short-read sequencing platforms, which necessitate deeper sequencing coverage and robust bioinformatics pipelines for accurate consensus generation. Additionally, the per-sample reagent cost can be higher than that of routine PCR assays [49, 50]. Regarding its developmental stage, ONT technology is mature and commercially available. Its application for MPXV genome sequencing has been rapidly adopted by public health laboratories worldwide and is now considered a routine tool for viral surveillance. This places ONT in an applied stage for public health purposes, though it is not yet widely deployed for primary clinical diagnosis [97].

Detection method based on immunology

While NAATs, such as PCR, offer excellent sensitivity and specificity for various common diseases, their widespread deployment is constrained by high costs, the requirement for technical expertise and specialized equipment, and the need for stringent contamination control to prevent false positives. These factors often render NAATs inaccessible to many healthcare facilities, particularly in resource-limited settings. In contrast, certain immunoassay-based methods, particularly those leveraging nanomaterials like LFAI and Raman scattering, present compelling advantages. These platforms are typically user-friendly, demand minimal specialized equipment, are cost-effective, rapid, sensitive, specific, and highly versatile [98]. Consequently, immunoassays hold substantial promise for the rapid, POCT of MPXV.

Lateral flow immunoassay

Recent advancements in LFAI have largely focused on developing sophisticated signal amplification strategies to overcome the inherent limitations of conventional colloidal gold nanoparticles (AuNPs). Although AuNPs are the most common reporters in LFAs due to their unique chemical, physical, and optical properties, and their relative ease of preparation and handling, they are often limited by low optical signal intensity and suboptimal sensitivity.[46] This inherent drawback has driven extensive exploration into advanced nanomaterials and signal enhancement technologies to significantly improve LFAI performance.

As a notable example, Wang et al. developed an enhanced LFAI system utilizing novel multilayered SiO_2-Au inner-core double quantum dot (QD) shell nanocomposites (termed SiO_2-Au/DQD), which effectively replaced traditional fluorescent (QD nanolabeling) and colorimetric nanolabels (AuNPs) [48]. These nanocomposites are engineered with a large SiO_2 core (~200 nm), a layer of precisely controlled-density 20 nm AuNPs, and thousands of smaller QDs (Fig. 5A), resulting in superior colloidal stability and enhanced detection sensitivity. This study successfully achieved specific screening of the MPXV antigen (A29L) within 15 min using a dual-signal output fluorescence immunoassay (Fig. 5B-C). Interestingly, the colorimetric readout of the SiO_2-Au/DQD-LFAI exhibited sensitivity comparable to that of traditional AuNP-based LFAI. However, the fluorescent signal-based detection mode demonstrated significantly higher overall 238 times more sensitive than ELISA and 3.3 times more sensitive than AuNP-based LFAI, respectively. This innovation marks a successful transition from a single-mode colorimetric detection to a more versatile and sensitive dual-mode system, crucially without sacrificing the operational simplicity required for POCT applications.

While the SiO_2-Au/DQD system represents a significant step forward in signal amplification, continuous innovations are emerging, not only targeting sensitivity but also enhancing operational simplicity and real-world applicability. Current efforts in MPXV LFA development remain centered on integrating novel nanomaterials to boost performance while retaining the core advantages of rapid antigen testing: simplicity, speed, and cost-effectiveness [46]. It is important to acknowledge that, even with these improvements, LFAs generally exhibit lower sensitivity than nucleic acid-based assays and are largely qualitative. Currently, several MPXV LFA prototypes

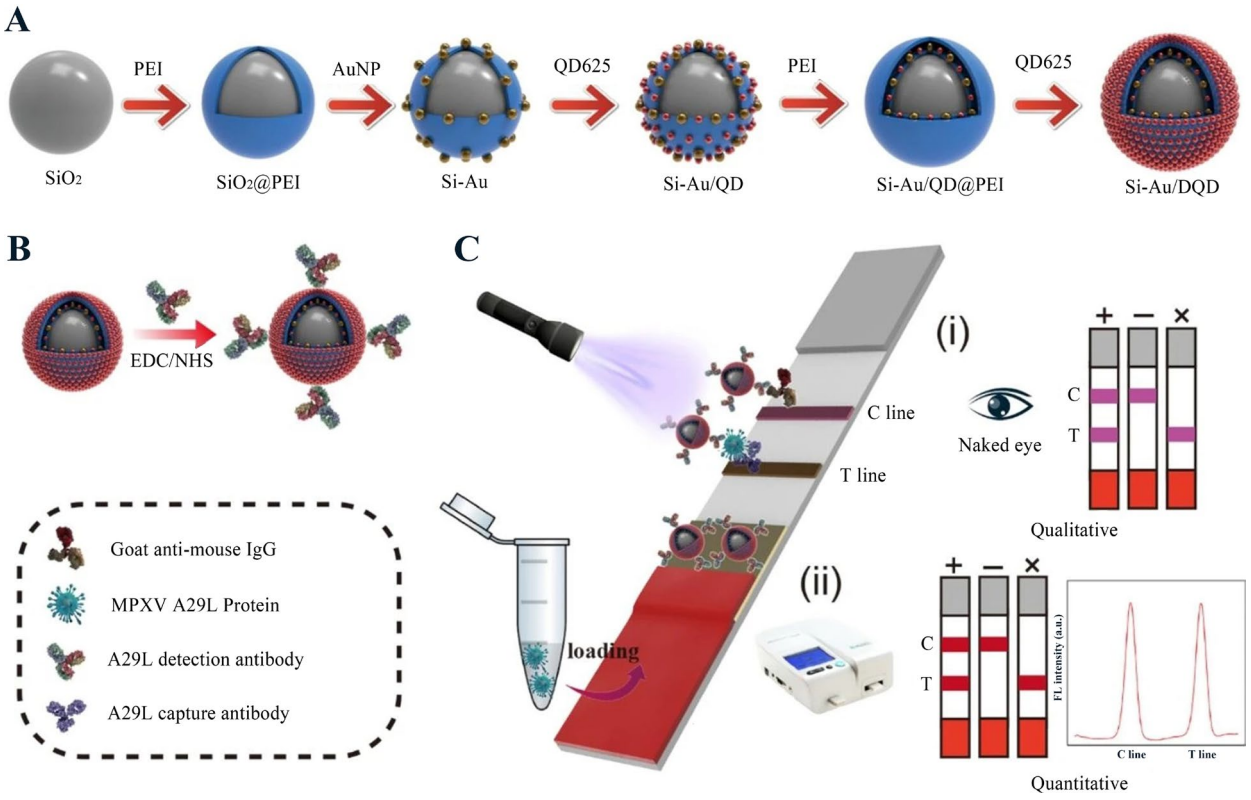


Fig. 5 Application of LFAI in MPXV detection. **A**) Process for the synthesis of Si-Au/DQDs NPs. **B**) Antibody coupling assay of MPXV A29L. **C**) Diagram of Si-Au/DQD-enabled LFAI analysis of MPXV A29L protein. **A**-**C**) Reproduced with permission [48]. Copyright 2023, Journal of Nanobiotechnology

are undergoing clinical evaluation, with some already deployed in outbreak settings for preliminary screening purposes [99]. These ongoing studies are pivotal for validating the real-world utility of advanced systems like SiO_2-Au/DQD and for guiding the future development of next-generation LFAs that optimally balance sensitivity with ease of use.

Raman scattering

Raman spectroscopy offers a promising approach to overcome the limitations of conventional fluorescent probes used for visualizing target proteins or molecules in living cells and tissues. These limitations include restricted color multiplexing, broad fluorescence spectra, susceptibility to photobleaching, and limited multiplexing capabilities [100]. Unlike fluorescent probes, Raman spectroscopy provides a molecule-independent detection mechanism that does not rely on intrinsic fluorescence properties, enabling label-free detection and imaging of biomolecules [51]. Furthermore, Raman scattering can be significantly enhanced through techniques such as resonance Raman scattering, which uses electrically excited resonance, or surface-enhanced Raman scattering (SERS), which leverages collective electronic oscillations in metallic nanostructures [51, 101]. SERS, in particular, has been widely applied for detecting viruses such as dengue [102], hepatitis B [103], and SARS[104] due to its high sensitivity, rapidity, simplicity, specificity, cost-effectiveness, and non-invasive nature. These attributes suggest that SERS holds significant potential for MPXV detection.

In a foundational application of SERS for MPXV detection, Zhang et al. developed a method combining SERS with calcium ions, iodide ions, and silver nanoparticles to detect a broad range of antigenic proteins and capture characteristic fingerprints of the MPXV genome without requiring specific probes (Fig. 6A) [105, 106]. This approach achieved a lower detection limit of 100 copies/mL, demonstrating a high signal-to-noise ratio and excellent reproducibility. By exploring the relationship between characteristic peak intensities and concentrations of proteins and nucleic acids (Fig. 6B-E), concentration-dependent spectra with robust linear correlations were established [107]. Additionally, principal component analysis (PCA) enabled the identification of SERS spectra for four distinct MPXV proteins in serum, highlighting the potential of this rapid assay for managing ongoing Mpox outbreaks and preparing for future epidemics.

However, signal interference in complex biological matrices remains a critical challenge for SERS applications. To address this, studies have explored the creation

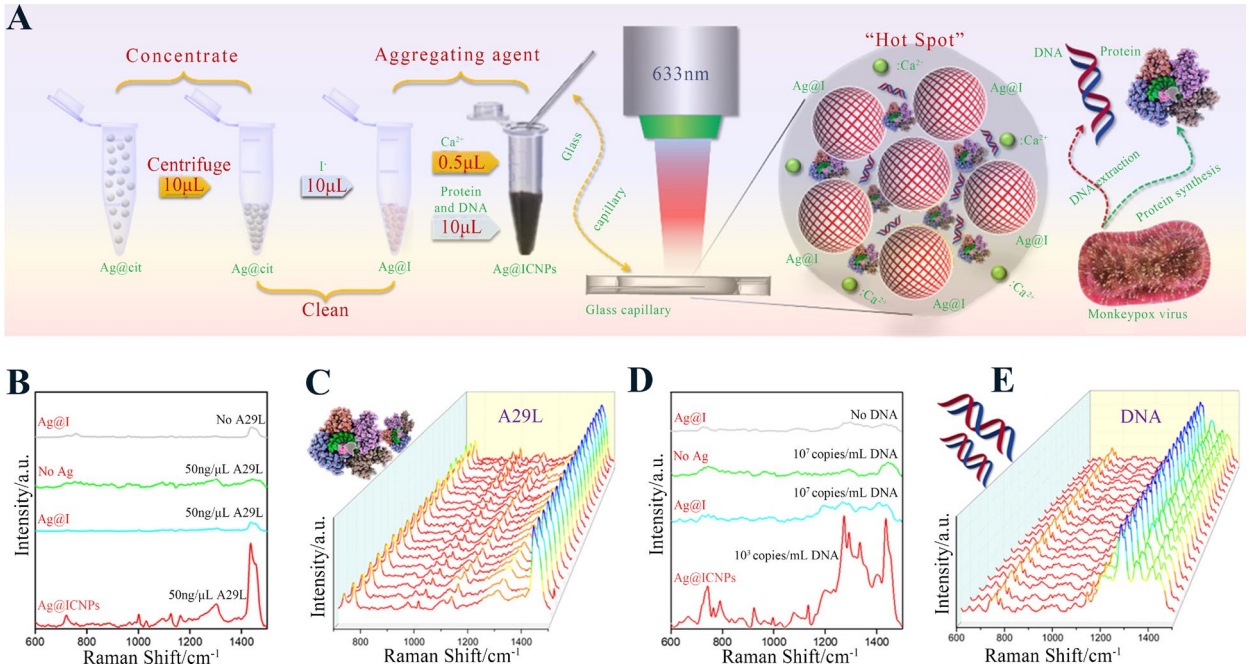


Fig. 6 Application of Raman scattering system in MPXV detection. **A**) Schematic conceptualization of silver-enhanced substrates and viral samples, the SERS assay, and the relationship between the "hotspots" created by the silver-enhanced substrates and the viral samples. **B**) SERS spectra of A29L in different systems. **C**) SERS spectra of 20 sets of randomly collected A29L (50 ng/μL) samples on the basis of method (Ag@ICNPs) and SERS spectra of 20 sets of randomly collected A29L (50 ng/μL) samples on the basis of method (Ag@Ag). **D**) MPXV DNA SERS spectra in different systems. E) SERS spectra acquired from 20 sets of randomly collected MPXV DNA (10^3 samples/mL) samples according to the method (Ag@ICNPs).**A**-**E**) Reproduced with permission [107]. Copyright 2023, American Chemical Society

of SERS hotspots for virus detection by aggregating silver nanoparticles, integrating PCA, and using sodium borohydride reduction to rapidly acquire MPXV fingerprints [78]. These fingerprints were successfully detected and characterized in both serum and artificial vaginal secretions [52]. Unlike the approach by Zhang et al., which focuses on direct detection of antigenic proteins and genomes, this strategy mitigates signal interference by actively designing hotspot structures, enhancing selectivity and achieving low detection limits for viral characterization and quantitative analysis [78]. These advancements underscore the potential of SERS as a robust, high-sensitivity tool for MPXV diagnostics in complex biological environments.

To further improve the accuracy, sensitivity, and applicability of surface-enhanced Raman scattering (SERS) for mpox virus (MPXV) detection, researchers have developed a novel colorimetric/SERS dual-signal immunochromatographic assay (ICA) method [108]. This approach utilizes molybdenum disulfide (MoS2) nanosheets coated with a 1-nm polyethyleneimine intermediate layer, enabling the electrostatic adsorption of two layers of AuNPs. This dual-layer configuration enhances colorimetric signals while generating a high density of effective SERS hotspots. By combining the chemical enhancement properties of MoS2 with the colorimetric capabilities of MoS2 and AuNPs, the MoS2@Au–Au nanostructure achieves synergistic improvements in both colorimetric and SERS detection, significantly enhancing the accuracy and usability of MPXV diagnostics. Unlike previous SERS strategies that focused on detecting proteins and genomes or optimizing hotspot structures, this method integrates SERS with the established ICA test strip platform, incorporating colorimetric readouts to simplify equipment requirements and improve operational convenience. This advancement represents a critical step toward meeting the practical demands of POCT.

Despite its promise for label-free, highly sensitive, and multiplexed pathogen detection, SERS faces several challenges [102]. Raman signals are susceptible to interference from other molecules in complex biological matrices, such as serum, which can compromise detection specificity [107]. Additionally, the reproducibility of SERS depends heavily on the consistent fabrication of nanostructured substrates to generate reliable hotspots. The need for sophisticated optical instrumentation and advanced data analysis algorithms further limits the portability and ease of use of SERS-based methods. Consequently, most SERS-based MPXV detection strategies remain in the proof-of-concept or fundamental research stage, requiring significant development to achieve robust, commercially viable diagnostic platforms. Continued efforts to address these technical hurdles will be

essential for translating SERS into practical, field-deployable diagnostic tools for MPXV.

Intelligent Mpox antigen detection based on nanomaterials

Advanced biosensors leveraging the unique physicochemical properties of nanomaterials are being developed for highly selective and sensitive virus detection. Among these, luminescent carbon quantum dots (CQDs) have garnered significant attention due to their high photoluminescence, large specific surface area, and good biocompatibility [109, 110]. CQDs can utilize the physical effects of nanostructures, such as plasmon resonance and energy transfer, to enhance local electromagnetic fields, thereby improving their luminescence efficiency or excitation rate for sensing applications.

Applying these concepts to Mpox detection, Yun et al. developed a novel electrochemical biosensor utilizing graphene quantum rods (GQRs). They first synthesized MoO_3-GQRs via a "bottom-up" approach. This nanocomposite was then electrodeposited onto carbon fiber paper (CFP) by chronoamperometry to form the sensor platform. An antibody specific to the Mpox A29 protein (AbA29) was subsequently immobilized onto the MoO_3-GQRs using chemical cross-linking, creating the final immuno-probe (Fig. 7A) [54, 111]. Detection of the A29 protein (A29P) was then performed using electrochemical methods such as differential pulse voltammetry (DPV), electrochemical impedance spectroscopy (EIS), and cyclic voltammetry (CV) (Fig. 7B, C) [112, 113].

The working principle for this type of biosensor is that the specific antigen–antibody binding on the electrode surface hinders the mass diffusion of the redox probe, causing a measurable change in the electrochemical signal. Pushing this technology further towards POCT, another biosensor was constructed using laser-scribed graphene (LSG), which can be fabricated directly onto a paper substrate [53]. This nano-biosensor platform consists of a porous graphene electrode modified with gold nanostructures (AuNS) and functionalized with the A29 monoclonal antibody (Fig. 7D) [114]. This design enables the sensitive detection of the A29 protein and MPXV. By integrating with a smartphone and a portable potentiostat (Fig. 7E), the system provides rapid, accurate, and selective electrochemical testing directly from samples without pretreatment (Fig. 7F,G). This integration represents a significant advancement towards a viable POCT diagnostic tool for MPXV.

Despite their promise, a key challenge for nanomaterial-based detection is achieving high efficiency at very low analyte concentrations. At ultra-low concentrations, the small number of target molecules results in extremely weak signals (e.g., fluorescence, Raman scattering) that are often obscured by instrument background noise, Rayleigh scattering, sample autofluorescence, or electrochemical background currents. Strategies to overcome this include sample pre-enrichment (e.g., using magnetic beads) or local field enhancement (e.g., leveraging plasmonic effects) to improve the signal-to-noise ratio and amplify the signal [115, 116]. Furthermore, at low concentrations, the binding probability between the probe and target is low, leading to slow reaction kinetics. Designing high-affinity protein receptors or novel DNA nanostructures as probes can significantly enhance binding efficiency and improve detection limits [117]. In summary, nanomaterial-based biosensors leverage the unique properties of nanomaterials to achieve highly sensitive and specific detection of MPXV antigens, often in miniaturized formats suitable for portable devices [53]. Key advantages include high theoretical sensitivity, design flexibility, and strong potential for POCT integration. The major challenges hindering their widespread adoption are concerns regarding the long-term stability of functionalized nanomaterials and their susceptibility to non-specific binding in complex biological matrices (e.g., serum, saliva). Virtually all biosensors of this type reported for MPXV detection are currently in the proof-of-concept or early prototype stage and confined to laboratory settings [118].

Novel therapeutic strategies for Mpox

Currently, there is no safe and effective treatment for Mpox. While antiviral drugs such as cidofovir, vaccinia immune globulin (VIG), and tecovirimat (ST-246) have received emergency use authorization from FDA for Mpox treatment, these agents were not developed specifically for Mpox, and their efficacy remains debated [119, 120]. Consequently, there is an urgent need to develop targeted, safe, and effective anti-Mpox therapies [119, 120]. In recent years, innovative approaches, including antibody-based therapies, adeno-associated virus (AAV)-delivered CRISPR antivirals, and nano therapy, have emerged as promising strategies (Table 2).

Antibody-based therapy

Antibody-based therapies have shown efficacy in treating various infectious diseases [127, 128]. and offer a promising avenue for managing severe mpox cases, particularly given the scarcity of specific MPXV treatments. Monoclonal antibodies, immunoglobulins, and convalescent plasma have been explored as adjuvant therapies [129, 130]. Early studies focused on neutralizing antibodies targeting specific MPXV proteins. For example, Li et al. demonstrated that three anti-MPXV monoclonal antibodies (9F8, 3A1, and 2D1) targeting distinct epitopes on the MPXV A29L protein exhibited strong synergistic antiviral activity in vitro (Fig. 8A-E) [92]. Animal studies further confirmed that a cocktail of these multi-epitope antibodies targeting A29L provided robust therapeutic

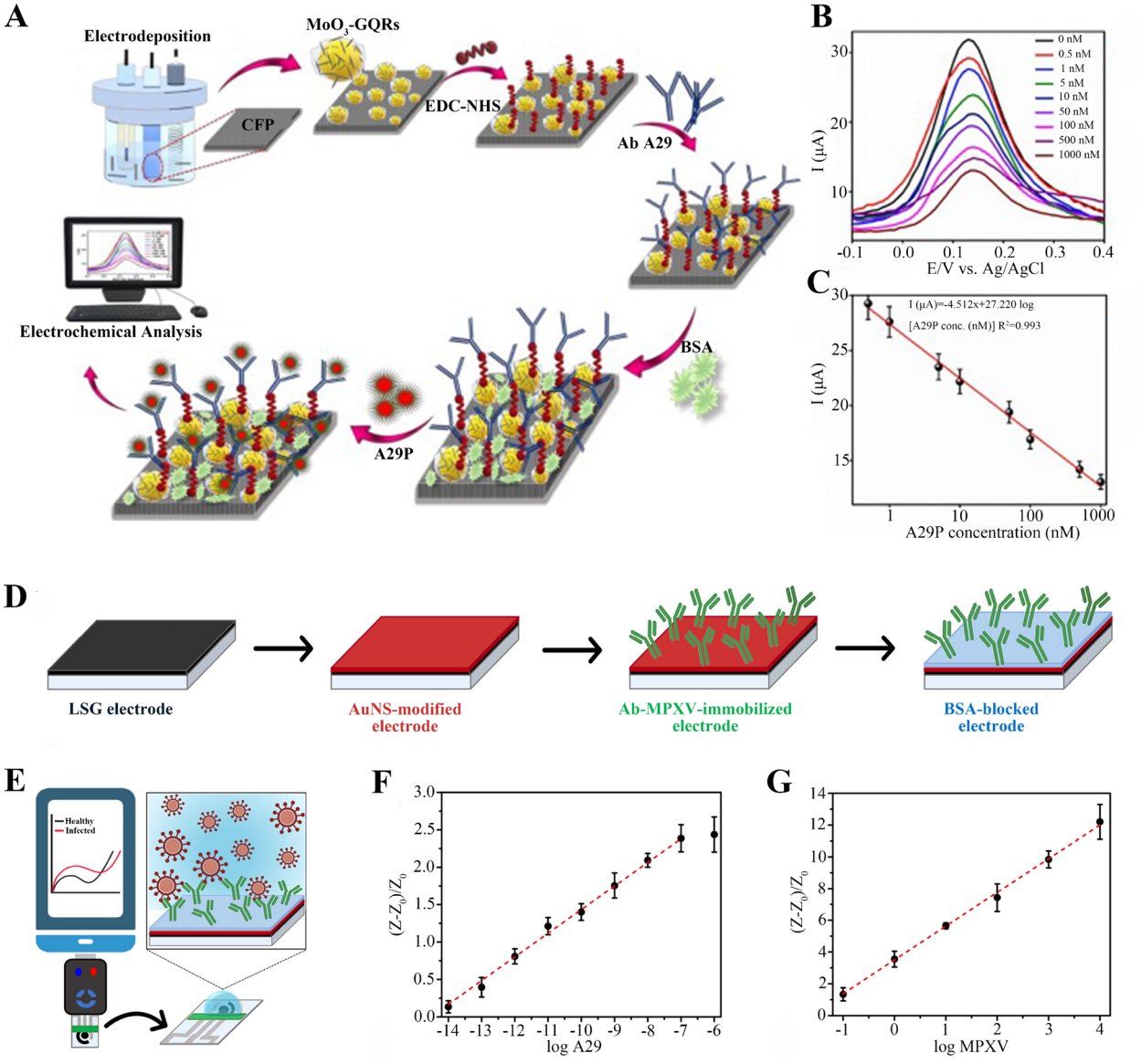


Fig. 7 Application of some nanotechnology in MPXV detection. **A**) Schematic representation of the label-free immunosensor. **B**) DPV response of Ab A29/MoO3-GQR prepared at different concentrations of A29P (0 nm to 1000 nM) used with 0.01 M PBS. **C**) Calibration plot of current response of DPV versus log concentration of A29P. **A**-**C**) Reproduced with permission [54]. Copyright 2024, Journal of Colloid and Interface Science. **D**) Characterizations of each functionalization step of the disposable nano biosensor. **E**) Schematic diagram of the MPXV electrochemical assay using a nano biosensor (LSG/AuNS/Ab-MPXV/BSA) connected to a portable micro-potentiometer controlled by a smartphone. **F**) Analysis curves of normalized RCT values from Nyquist plots and the log of A29 protein concentration. **G**) Analytical curve obtained from normalized RCT values extracted from Nyquist plots as a function of the logarithm of the MPXV viral loads. **D**-**G**) Reproduced with permission. [53] Copyright 2023, American Chemical Society

efficacy (Fig. 8F-G), establishing a proof-of-concept for antibody-based mpox therapy [121].

Recognizing the risk of viral escape from single-target antibody therapies and the potential for cross-protection among orthopoxviruses, recent research has shifted toward antibodies with broader neutralizing capabilities. Gil Chuk et al. isolated a panel of human monoclonal antibodies from individuals vaccinated against or infected with orthopoxviruses, identifying antibodies targeting the A33, L1, A27, and H3 antigens with potent neutralizing activity against both VACV and MPXV in vitro [130, 131]. Combinations of these high-potency monoclonal antibodies demonstrated superior protection against VACV compared to VIG in preclinical models, highlighting a strategic shift from targeting multiple epitopes on a single protein to broad-spectrum neutralization of multiple conserved viral surface proteins [132, 133]. This approach lays the groundwork for universal therapies capable of addressing viral mutations and providing efficacy across orthopoxviruses, potentially

Table 2 Advanced techniques for mpox treatment

Treatment strategy	Mechanism of action/Main method	Advantage	Limitation	Applicable scene	Reference
Antiviral drug	Inhibit viral replication	Shorten the course of disease and effective for severe patients	Lack of specific drugs, poor curative effect	Early infection, severe cases	[119, 120]
Antibody-based therapy	Antigen and antibody bind	Directly neutralize the virus; Rapid onset of effect; It is effective for severe patients	Antibody has low stability and high preparation cost	Emergency treatment for severe cases	[121, 122]
CRISPR	Targeted cutting of viral genome	Potential broad-spectrum antiviral capability, can be designed to target conserved areas	Off-target risk and immune escape	Precision therapy	[123–125]
Nano therapy	Photothermal therapy and photodynamic therapy	Highly effective virus killing, high biocompatibility	Requires precise lighting control, long-term safety to be verified	Treatment of local skin lesions	[25, 126]

CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats.

outperforming traditional antivirals like VIG and cidofovir [132, 133].

Despite their promise, antibody therapies face challenges, including in vivo stability issues, as antibodies are susceptible to degradation by enzymes and chemicals in physiological environments [134]. Additionally, some antibody fragments are also rapidly metabolized, and adverse events such as cytokine release syndrome (CRS) or organ toxicity, some of which are even life-threatening, can limit their use [122, 135]. Key limitations also include high production costs, the potential for viral escape mutations, the need for intravenous administration, and the risk of adverse events. Optimizing antibody delivery systems, such as through advanced stabilization techniques or alternative administration routes, may address these challenges and enhance the clinical utility of antibody-based mpox therapies [129, 130]. Currently, several MPXV-specific monoclonal antibody cocktails have shown efficacy in preclinical animal models, positioning them in advanced preclinical development stages [136].

CRISPR

The CRISPR-Cas system functions by inducing precise double-strand DNA breaks (DSBs) at specific genomic loci, which are targeted by single-guide RNAs (sgRNAs) [137]. This gene-editing mechanism has been extensively explored for antiviral therapies against pathogens such as hepatitis viruses[138, 139] and herpes viruses [140, 141]. In the context of Orthopoxviruses, Siegrist et al. utilized CRISPR-Cas9 to engineer sgRNAs targeting three conserved VACV genes: A17L, E3L, and I2L [125]. This multi-target approach was designed to ensure redundancy and robustly inactivate essential viral genes, thereby inhibiting viral replication. After verifying the CRISPR target, the team employed an adeno associated virus (AAV) as a delivery vector for SaCas9 and the sgRNAs to assess the safety and efficacy of this strategy. The AAV-mediated delivery of this CRISPR-based antiviral, even when targeting just a single gene, reduced viral titers by up to 92.97%. Although the CRISPR-Cas9 system has been successfully used in several antiviral therapies, there are limitations that need to be considered before it can be used in clinical trials. The most critical challenge of CRISPR technology is the potential off-target effect, which can induce mutations or chromosomal translocations [142]. The off-target effect of CRISPR technology can be markedly improved by the use of a paired Cas9 cleavage enzyme [123]. Another limitation of CRISPR technology is immune escape. Another crucial limitation of technology is the immune escape of the virus [124]. A poxvirus that mutates at a site in the sgRNA target can cause that CRISPR target to fail. Modifying sgRNAs and setting up CRISPR multi-targets may be the key to solving this problem.

Nano therapy

Nanomaterials have garnered significant attention in biomedicine, particularly for antiviral drug delivery, due to their unique properties, including small size (enhancing bioavailability and controlled release), tunable surface charge (enabling encapsulation of diverse drugs), and high surface area to volume ratio (improving the solubility) [143, 144]. Gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs) have demonstrated efficacy in inhibiting MPXV infection and transmission [145, 146]. For instance, studies have shown that AgNPs block vaccinia virus infection by interfering with viral entry through a macrophage-dependent mechanism [147, 148]. However, the biodegradability and biocompatibility of these inorganic nanomaterials require further investigation to ensure clinical safety.

In contrast, organic nanomaterials with aggregation-induced emission luminogen (AIEgen) properties offer unique advantages for integrated diagnostics and therapeutics. AIEgen is considered to be a very promising photosensitizer in the field of fluorescence [149, 150], and it has been utilized for detecting various pathogens, including specific detection of bacteria, virus and cancer

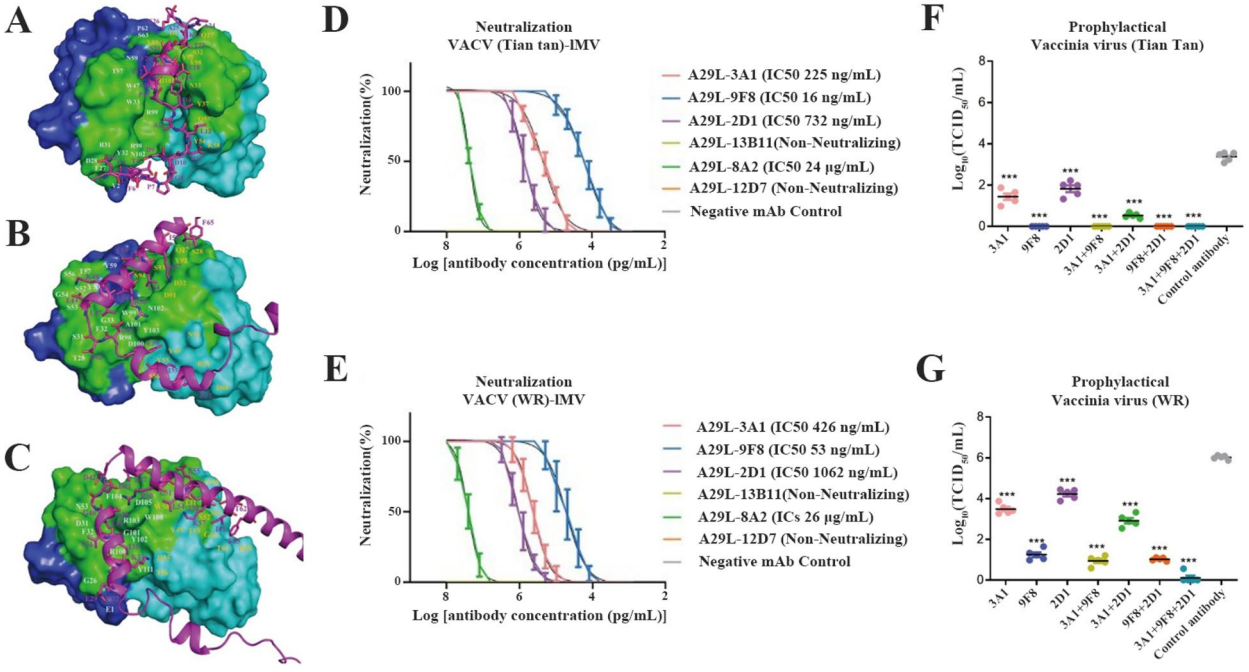


Fig. 8 Antiviral effects in vitro and in vivo and the interface of MPXV A29L protein-specific antibodies 3A1, 9F8, and 2D1. **A**-**C**) Interfaces of A29L protein-9F8 (**A**), A29L protein-2D1 (**B**) and A29L protein-3A1 (**C**). The Fv domains of the antibodies are shown in surface representation with the heavy chain, light chain and CDRs colored in blue, cyan and green, respectively. The residues on the heavy chain and light chain that are involved in A29L protein binding are labelled in white and orange. A29L protein is shown in cartoon and colored in magenta. The residues involved in 9F8, 2D1 or 3A1 binding are shown in sticks. **D**-**E**) Synergetic microneutralization ability of MPXV A29L protein specific antibodies 3A1,9F8 and 2D1 against IMV form of VACV Tian tan strain (**D**) and IMV form of VACA WR strain (**E**). **F**-**G**) The virus titers in the lungs of the mice treated with each antibody prophylactically were determined on four days after infection, the t-test was used for comparisons between groups. ***$p < 0.001$ versus the negative control group. **A**-**G**) Reproduced with permission [121]. Copyright 2023, Emerging Microbes & Infections

cells [13, 151–154]. Additionally, AIEgen with specific molecular configuration has photodynamic and photothermal properties, and can be used as a therapeutic agent in some cases.[155] It has been widely used in the treatment of many diseases, including viruses, cancer and bacterial infections [18, 19, 148, 156, 157]. TPE-BT-DPTQ, which is a high-performance photothermal molecule, was encapsulated by Liao et al. with AIEgen in macrophage membrane to prepare a new type of cell membrane-based biomimetic nanoparticles (TBD@MNPs) (Fig. 9A) [126]. The virus has a strong binding ability to macrophage membranes. After co-incubation with TBD@MNPs for 10 min (Fig. 9B), confirming that TBD@MNPs can target poxvirus particles. Under the irradiation of 808 nm laser, the photothermal effect was significantly activated and targeted to kill virus particles, which significantly healed the infected wound in the tail of tail mice (Fig. 9C) [158]. Because of the efficient fluorescence imaging ability of TBD@MNPs, this method also realized the accurate tracking of lesions. Inoculation of diseased tissue suspension did not cause inflammation or caudal infection in healthy mice, thus successfully blocking the transmission of the virus. This study is the first to demonstrate the therapeutic use of nanomedicine for Mpox, but its therapeutic mechanism mainly relies solely on a single photothermal therapy (PTT).

To address the limitations of single-modality treatment and enhance therapeutic efficacy, an advanced iteration was developed. Building on TBD@MNPs, researchers pre-activated macrophage membranes with vaccinia virus and encapsulated them around polymer nanoparticles loaded with a multifunctional AIEgen photosensitizer, TPE-BTDCTBT, to create PN-AIEM nanoparticles (Fig. 9D) [25]. Virus-activated macrophage membranes express higher levels of receptors, enhancing viral recognition and clearance. PN-AIE MΦ nanoparticles exhibit near-infrared II (NIR-II) fluorescence, photothermal, and oxygen-independent photodynamic properties, enabling precise binding to viral receptors, prolonged retention in infected skin lesions via intravenous injection, and effective imaging and treatment under 808 nm laser irradiation (Fig. 9E,F). This strategy synergistically combines photodynamic therapy (PDT) and PTT to eliminate viruses and promote wound healing in mouse tail models [159]. The transition from single-modality PTT to a combined PTT/PDT approach marks a significant advancement in AIEgen-based nanomedicine, offering a multifunctional platform for mpox treatment and diagnostics. Remarkably, the combined application of

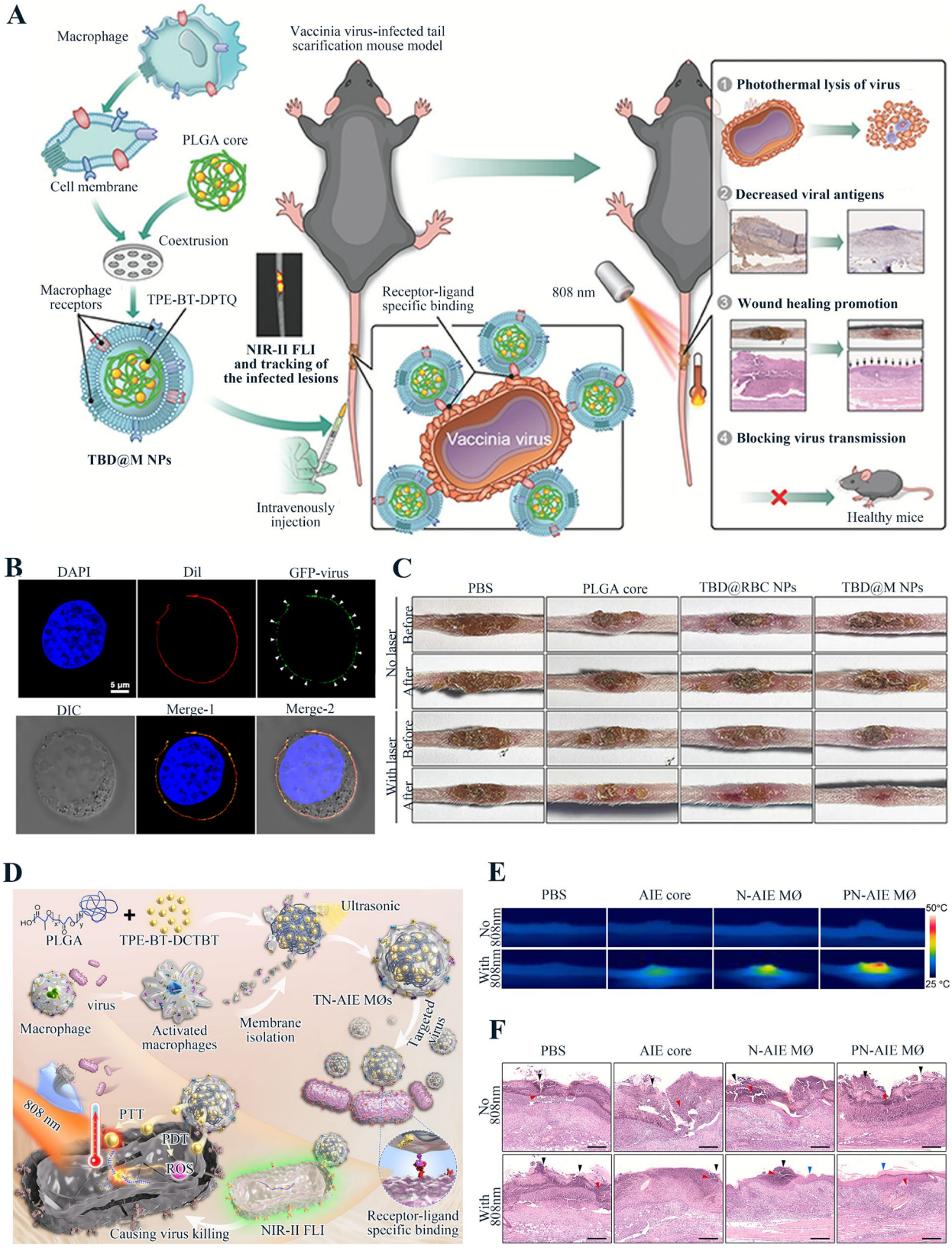


Fig. 9 (See legend on next page.)

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Fig. 9 Application of AIEgen in MPXV detection and killing. **A**) Schematic diagram of macrophage-like nanoparticles (TBD@MNPs) based of aggregation-induced emission (AIE) to detect and kill MPXV. **B**) Confocal images of GFP-rabies virus (green) bound to macrophage membranes surface after 2 min of virus co-incubation with macrophages. The nuclei and macrophage membrane are stained with DAPI (blue) and DiI (red) respectively. Bound virus particles are marked with white arrows. **C**) Surface view of tail infection before and after the different interventions. **A**-**C**) Reproduced with permission [126]. Copyright 2023, Wiley–VCH GmbH. **D**) Schematic diagram of PN-AIE MØ for killing and detecting of MPXV. **E**) Fluorescence imaging of infected skin lesions in mice. **F**) PN-AIE MØ obviously healed the wound in the tail of the mouse with laser. **D**-**F**) Reproduced with permission [25]. Copyright 2024, Elsevier Inc

PTT and PDT had not been previously explored for the treatment of viral infections like Mpox. Our investigation highlights a significant breakthrough in this domain, demonstrating the feasibility and substantial promise of this combined strategy. Specifically, the two studies discussed above (referring to the prior section's content, which would likely be about AIEgen-based nanoparticles) innovated by leveraging the versatility of AIEgen integrated within macrophage membrane coated nanoparticles for targeted Orthopoxvirus elimination [160, 161]. This breakthrough in exploring biomimetic nanoparticles carries profound practical significance for controlling the emergence and spread of MPXV, particularly in the context of a pandemic.

This type of nanotherapy, especially those employing AIEgen-based materials, introduces a novel "theranostic" strategy that synergistically combines targeted virus elimination with advanced imaging capabilities [25]. The key advantages of these systems stem from their high specificity, achieved through biomimetic macrophage membrane coating, and efficient pathogen inactivation via potent photothermal and/or photodynamic effects. However, significant challenges persist, including the inherently limited tissue penetration depth of light, which restricts treatment efficacy to superficial or readily accessible lesions. Furthermore, the potential for long-term toxicity and complex biodistribution profiles of these nanomaterials necessitates comprehensive preclinical and clinical evaluation. While the pioneering work on AIEgen nanoparticles for MPXV treatment is exceptionally promising and has demonstrated efficacy in mouse models, it remains firmly within the preclinical development stage. Before clinical translation, rigorous pharmacological and safety studies, particularly in non-human primate models, are essential.

The prevention of MPXV

Currently, no vaccine with high specificity and efficacy against MPXV exists. Historically, smallpox (variola) vaccines were the primary line of defense. Until 2015, ACAM2000, a live vaccinia virus (a related Orthopoxvirus) vaccine, was the only smallpox vaccine licensed by FDA and showed cross-protective efficacy against MPXV. In 2019, the FDA approved JYNNEOS (Modified Vaccinia Ankara, MVA), administered as a two-dose subcutaneous series (0.5 mL per dose, 4 weeks apart), for the prevention of both smallpox and Mpox [162, 163].

These existing vaccines confer protection through cross-immunization. However, they are not specific for MPXV and, consequently, demonstrate limited protective efficacy against MPXV. Furthermore, routine smallpox vaccination was discontinued globally in 1980 [164, 165]. Although emergency vaccination has resumed, the available vaccine supply remains insufficient. This shortfall underscores the urgent priority for developing novel, targeted vaccines against MPXV infection. In response, next-generation Mpox vaccines are in development, including DNA vaccines, RNA vaccines (Table 3), multi-epitope vaccines, and nanoparticle-based antigen carrier vaccines. These new approaches aim for high specificity and robust immunogenicity [29, 166, 167], offering more effective and targeted solutions for Mpox vaccine development.

RNA vaccine

Developing Mpox specific vaccines is a critical goal, and mRNA vaccines may also be the key to preventing Mpox. The proteome of the Orthopoxvirus is large and complex, encoding over 200 types of proteins [130]. During the infection process, the virus exists in two different antigen forms, namely mature virion (MV) or enveloped virion (EV), which contain 25 or 6 surface proteins, respectively [172]. In previous studies, intracellular mature virus (IMV) specific proteins and extracellular enveloped virus (EEV) specific proteins, such as A29L, E8L, M1R, A35R, B6R, L1R, A27L, A33R, A29 L, H3 L, A5L, A4, A56, F9, H3, and B5R [166, 168, 173], have been experimentally proven to be immunogenic and can protect mice from VACV infection. For example, Fang et al. designed MPXVac-0976, a multivalent mRNA candidate vaccine for Mpox. This construct utilized five MPXV antigens (A29L, E8L, M1R, A35R and B6R), which were linked in tandem by 2A peptides and codon optimized(Fig. 10A) [166]. They found that the multivalent vaccine demonstrated good immunogenicity and induced high levels of neutralizing antibodies in mice. (Fig. 10B), providing protection against Mpox attacks. Furthermore, given its simpler production process, antigen tandem co-expression is an attractive direction. However, by concatenating the five MPXV antigens through a 2A peptide segment, only antibodies against A35 R and E8 L were induced, which could not induce antibodies against all antigen proteins, thus affecting the immune efficacy of MPXVac-097 to some extent.

Table 3 DNA vaccine and RNA vaccine of Mpox

Type	Name	Disease	Status	Antigen	Motivations	Reference
RNA vaccine	MPXVac-097	MPOX	preclinical	A29L、E8L、M1R、A35R、B6R	Induced immunity	[166]
RNA vaccine	Rmix4、Rmix6	MPOX	preclinical	M1、H3、A29、E8、B6、A35	Induced immunity	[168]
RNA vaccine		MPOX	preclinical	A35 R、B6 R、A29 L、H3 L、E8 L、M1 R	Induced immunity	[28]
RNA vaccine	cirA29L、cirA35R、cirB6R 、cirM1R	MPOX	preclinical	A29L、A35R、B6R 、 M1R	Induced immunity	[169]
DNA vaccine		MPOX	preclinical	A5L、A15L、A35R、 B6R	Induced immunity	[167]
DNA vaccine		MPOX	preclinical	A4、A56、F9、H3、A27、A33、B5、L1	Induced immunity	[170]
DNA vaccine	4pox	MPOX	preclinical	L1、A27、B5、A33	Induced immunity	[171]

Abbreviations: MPOX, monkeypox.

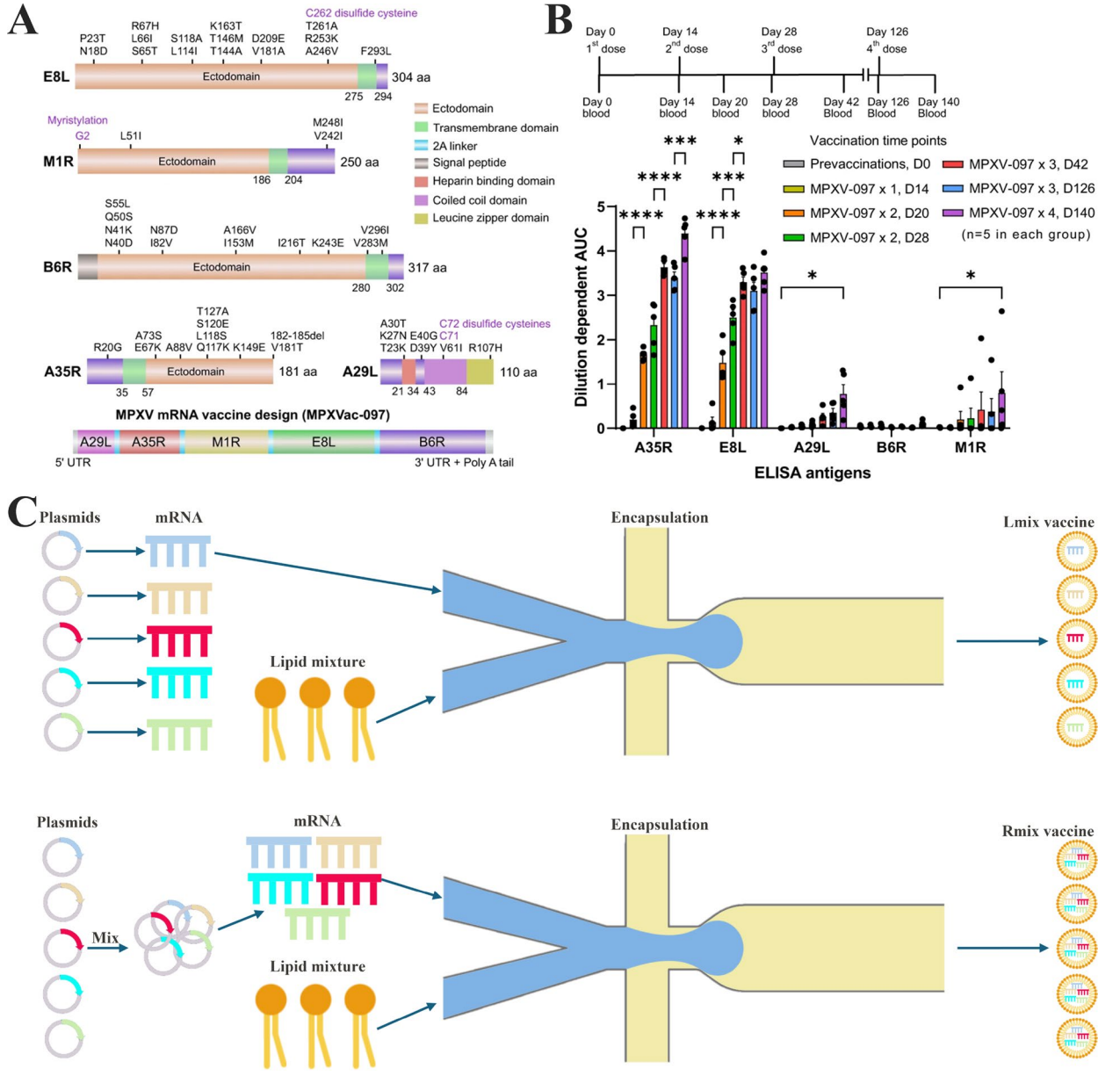

Fig. 10 Antibody responses induced by MPXVac-097 and B6, A35, A29, E8, M1 or H3 mRNA vaccines in mice and construction of Mpox multi-antigen mRNA vaccine candidates. **A**) Five neutralizing targets of MPXV mRNA vaccine candidate are connected by 2A linkers and translated from the same mRNA transcript, MPXVac-097. Antigen sequence difference between MPXV and MVA is shown. **B**) After being immunized with MPXVac-097 LNP-mRNA, mice showed significantly increased antibody titers against A35R and E8L (n = 5). A-B) Reproduced with permission [166]. Copyright 2023, Cell Research. **C**) Schematic diagram of formulation procedure for multi-antigen mRNA vaccine candidates

To address the issue of incomplete immunogenicity caused by tandem design, Zeng et al. adopted another technical approach, they developed quadrivalent (M1, A29, B6, A35, known as Rmix4) or hexavalent (M1, H3, A29, E8, B6, A35, known as Rmix6) polyantigen mRNA candidate vaccines by a simplified manufacturing strategy to mix the plasmids in equal amounts and transcribe them as a whole into mRNA, and then wrapped them with lipids to make candidate vaccine preparations(Fig. 10C) [168]. All mRNA components in the Rmix4/6 formula express all required antigen proteins and induce antibodies against all antigen protein. However, the Rmix4/6 study did not deeply verify the vaccine efficacy of all antigen combinations and failed to answer the key question of which antigen combination could produce the best immune effect.

In order to systematically screen and optimize antigen combinations, Zhang et al. designed and tested four formulations with combinations of two EV antigens (A35 R and B6 R), two MV antigens (A29 L and H3 L), four MV antigens (A29 L, H3 L, E8 L, and M1 R), and six EV + MV antigens (A35 R, B6 R, A29 L, H3 L, E8 L, and M1 R) (Fig. 11A) [28]. The cumulative potential of each immunogen in generating an immune response and eliminating VACV infection was found, that means a higher number of immunogen contributed to a more robust overall IgG response and associated neutralizing activity against VACV. Among them, the EV and MV antigen combination vaccines provided the strongest protection. This work clarified the advantages of multivalent antigen combinations and proposed excellent antigen combinations.

Beyond antigen selection and combination strategies, the molecular form of mRNA vaccines critically influences their efficacy and applicability. Conventional linear mRNA vaccines typically necessitate extensive base modifications to mitigate their inherent immunogenicity. They also suffer from relative instability and are prone to degradation at room temperature, which collectively limits their efficacy and broad applicability [174, 175]. In contrast, circular RNA (circRNA), owing to its closed-loop structure, exhibits lower innate immunogenicity (without the need for extensive modification), superior stability, and reduced cytotoxicity compared to linear mRNA [176–179]. This presents a compelling new avenue for overcoming the shortcomings of linear mRNA. Building on this, Zhou et al. leveraged an efficient and scalable circRNA engineering platform to develop four monovalent circRNA vaccines (cirA29L, cirA35R, cirB6R, and cirM1R) and a tetravalent hybrid vaccine, cirMix4 (Fig. 11B) [169]. They observed that cirM1R induced high antibody titers in mice, indicating its potential to elicit a robust humoral immune response. On the contrary, cirA35R notably stimulated significant T cell responses, suggesting its capacity to trigger effective cellular immunity. Furthermore, the multivalent cirMix4 vaccine successfully induced high-level neutralizing antibodies and cellular immune responses against both VACV and MPXV in mice. However, current circRNA vaccine strategies are primarily focused on monovalent designs. Embedding multiple antigen expression sequences into a single circRNA or encapsulating multiple circRNA expressing different antigens in the same liposome to achieve multivalent antigen expression may be the main development direction of circular RNA vaccines in the future.

Overall, RNA vaccines demonstrate promising immunogenicity, capable of eliciting robust T cell responses and high levels of neutralizing antibodies in preclinical mouse models. Nevertheless, current mRNA vaccines also have limitations of high innate immunogenicity and increased toxicity partly from lipid nanoparticle (LNP) formulations. Extensive alkalization modification and the development of less toxic lipid nanoparticles may be the key to solving this problem. Numerous multivalent MPXV mRNA vaccine candidates have shown robust protection in preclinical animal models, positioning them in the advanced preclinical stage. They are poised to enter clinical trials pending further development and regulatory approval.

DNA vaccine

Research on DNA vaccines has shown certain advantages similar to mRNA vaccines, including rapid production timelines and high stability,[180, 181] often at a lower manufacturing cost. Wang et al. designed a MPXV DNA vaccine encoding four antigenic protein sequences (A5L, A15L, A35R, and B6R) and optimized the final structure (Fig. 12A). Immune simulation analysis shows that this candidate vaccine could elicit effective cellular(Fig. 12 B) and humoral(Fig. 12 C) responses in the development of potential universal multi-epitope DNA vaccines [167]. However, this research only remains at the level of computer simulation, and its safety and effectiveness urgently need to be verified through in vivo experiments.

To provide this necessity in vivo validation, Hirao et al. advanced this research to animal models. They immunized macaques with DNA vaccines encoding VACV-A4, A56, F9, H3, A27, A33, B5, and L1-8 antigens, and administered intradermal (ID) or intramuscular (IM) injection to 14 macaques. After monitoring the degree, quality, and effectiveness of the vaccine induced response, it was ultimately found that the vaccine can cause extensive and powerful binding and neutralizing antibody reactions, as well as strong cellular immunity, resulting in a good level of immunity [182]. The combination of immune responses can greatly affect the challenge of macaques to deadly poxviruses, and multivalent DNA vaccines can effectively protect against MPXV infection. This

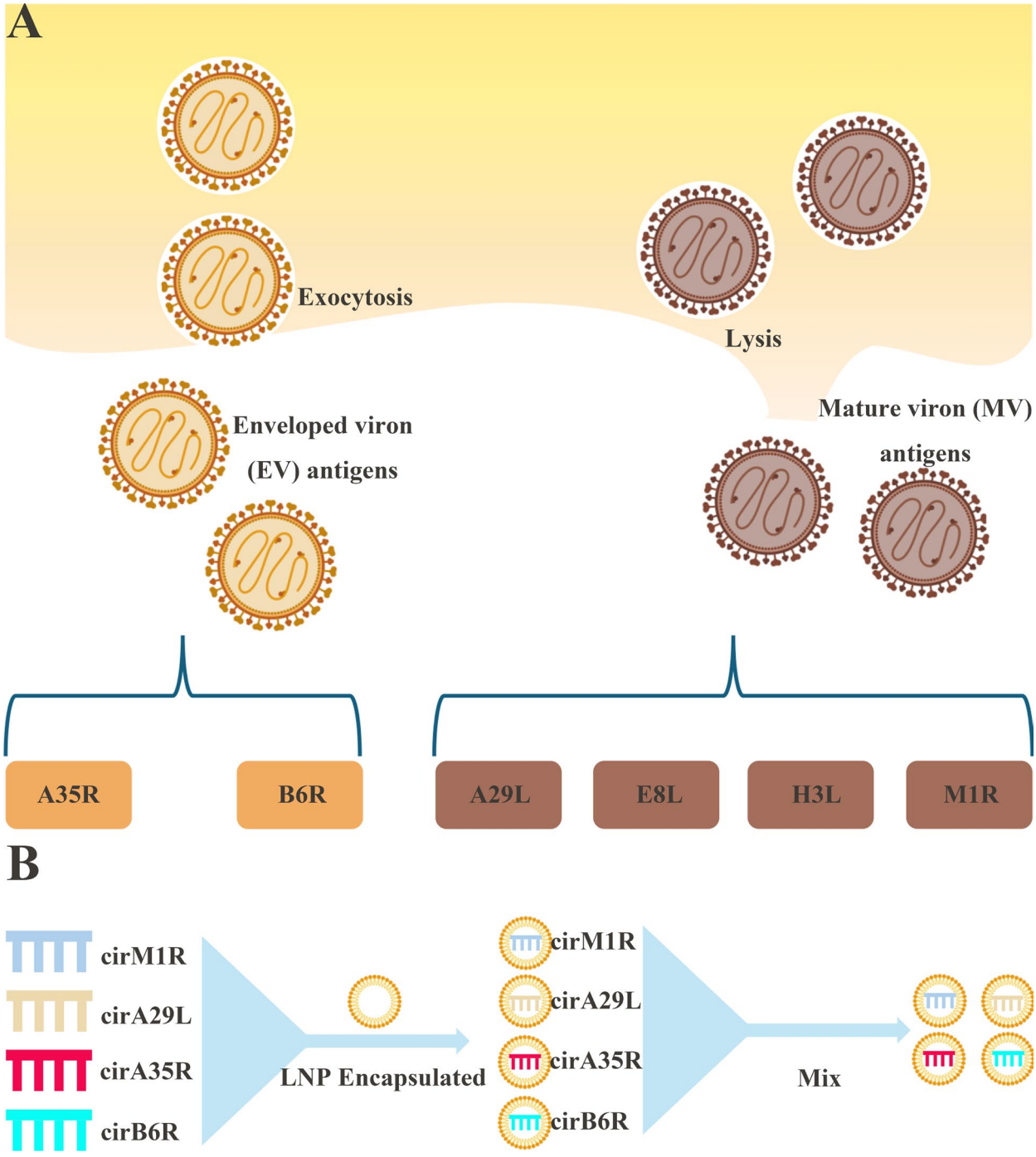

Fig. 11 Design of mRNA vaccine. **A**) Design of mRNAs encoding the multiple surface antigens from MPV. The full-length sequences of A35R, B6R and A29L were used with addition of 5′ and 3′ UTR sequence. For E8L, H3L and M1R, regions encoding their extracellular domains were fused to the N-terminal signal peptide tPA. tPA, human tissue plasminogen activator signal peptide. **B**) Schematic diagram of univalent vaccine (cirA29L, cirA35R, cirB6R and cirM1R) and quadrivalent hybrid vaccine cirMix4

phenomenon has great prospects for using DNA vaccine technology to prevent and treat emerging infectious diseases [170, 183]. However, DNA vaccines with ID or IM have shown poor immune efficacy in some clinical trials [184]. To address the bottleneck of weak immunogenicity in traditional injection methods, the research has shifted to using physical means to enhance the efficiency of vaccine delivery. Alan et al. reported effective delivery of a smallpox virus DNA vaccine using a new method, which involves skin electroporation using a microneedle array coated with plasmid DNA [185], This approach first involves drying the plasmid DNA to the tip of the

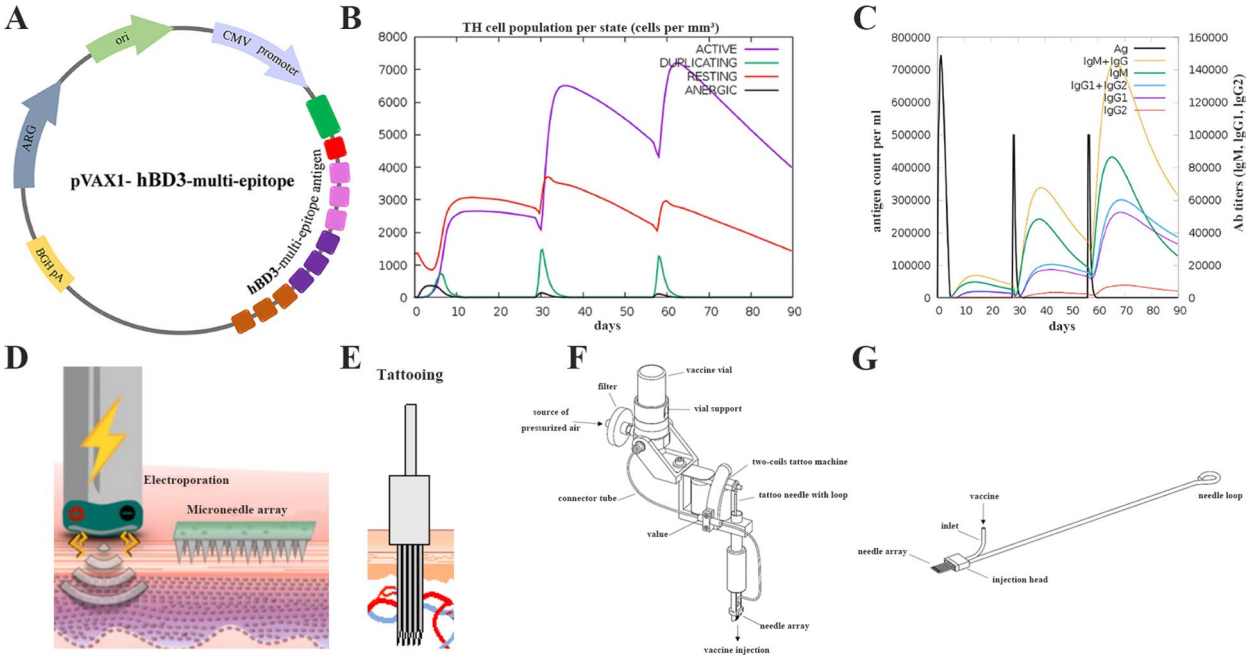

Fig. 12 Schematic diagram and its resultant humoral and cellular immunity of multi-epitope DNA vaccine construction and techniques to enhance the efficacy of the vaccine. **A**) Scheme of plasmid DNA containing multi-epitope antigen of the mpox virus. **B**) TH cell production due to exposure to antigen. **C**) Augmented antibody production. Immunoglobulin subclasses are indicated in different colors. **A**-**C**) Reproduced with permission [167]. Copyright 2023, Frontiers in Microbiology. **D**) Schematic representation of the electroporation and microneedle array. Reproduced with permission. [193] Copyright 2023, Journal of Molecular Biology. **E**) Schematic representation of DNA tattooing and microneedle array. Reproduced with permission. [194] Copyright 2020, Vaccines. F) Concept drawing showing the main components of IONAID. **G**) Close up of a needle array composed of 11 hypodermic needles. **F**-**G**) Reproduced with permission. [192] Copyright 2024, NPJ Vaccines

microneedle [186, 187]. These microneedles (≤ 1 mm in length) are inserted into the skin, DNA is dissolved in interstitial fluid, and then introduced into the surrounding cells through electroporation, thus injecting vaccines from the skin to enhance its effect(Fig. 12D). This strategy has been refined into minimally invasive skin electroporation microarray capable of promoting polyvalent immunity by delivering antigens encoded by eight different DNA plasmids simultaneously [170]. In addition to electroporation, DNA tattooing is another physical method aimed at enhancing intradermal immunity. This method involves injecting the vaccine formulation through tens of thousands of skin punctures to expand the contact area between the vaccine and immune cells in the epidermis, thereby triggering a strong cellular and humoral immune response (Fig. 12E) [188, 189], However, only the use of high doses of DNA for tattoo delivery, intensive tattooing protocols, or the use of large amounts of adjuvants can produce better immune effects [190, 191]. In order to solve the problems of excessive DNA dosage and complex tattooing conditions for DNA tattooing, a team developed an intradermal oscillating needle array injection device (IONAID) (Fig. 12F-G),[192] which can microinject any aqueous vaccine of controlled dose into the intradermal space, and verified the improvement of vaccine effect brought by this strategy. And ion-assisted DNA vaccines encoding the Ebola virus glycoprotein produced better T and B cell responses in mice.

In conclusion, DNA vaccines share the advantages of rapid production and high stability with mRNA vaccines, often at a lower cost. A historical limitation has been their relatively poor immunogenicity in humans when administered via conventional intramuscular injection. This can be overcome by advanced delivery methods like electroporation or tattooing, which enhance cellular uptake and immune activation. In addition, Several MPXV DNA vaccine candidates have demonstrated efficacy in non-human primate models, placing them in the advanced preclinical development stage.

Multiple epitope vaccine

Multiple epitope vaccines are computationally designed constructs, developed using immunoinformatic, that target multiple protein epitopes from a pathogen like MPXV [133]. By focusing on specific epitopes, they offer potential advantages such as lower allergenicity, reduced toxicity, and high immunogenicity, capable of inducing targeted, strong immune responses. The design process is a multi-step in silico workflow: (1) Epitope Selection: Predicting B-cell, helper T-lymphocyte (HTL), and cytotoxic T-lymphocyte (CTL) epitopes from the MPXV proteome, followed by screening for antigenicity, non-allergenicity,

and non-toxicity. (2) Vaccine Construction: Linking the selected epitopes with appropriate linkers and often fusing them with an adjuvant to enhance immunogenicity. (3) Preclinical Validation: Computationally validating the construction, which includes predicting secondary and tertiary structures, performing molecular docking with immune receptors (e.g., TLRs), and running molecular dynamics and immune simulations to assess stability and potential efficacy [195–197].

In the early designs of multi-epitope vaccines, the research focus tended to target a single type of immune response. Ezzemani et al. designed a multiple epitope subunit vaccine for preventing MPXV infection by connecting HTL/CTL epitopes like VACV and MPXV using immunoinformatic methods. The tightness of the specific binding between multi epitope vaccines and Toll like receptor 9 (TLR9) was demonstrated through molecular docking experiments, and the stability of the interaction was confirmed through molecular dynamics simulations. Immune simulations also indicate that the vaccine can induce effective immune responses against MPXV, but mainly through cellular immunity. Codon optimization and computer simulation studies have also shown its expression potential in the Escherichia coli K12 system. In summary, various data indicate that this vaccine is effective against MPXV. However, its immune response type mainly leans towards cellular immunity. This work demonstrates the potential of multi-epitope vaccine design, but the types of immune responses it triggers are relatively simple [198]. To make up for the deficiency of the above-mentioned vaccines in inducing a comprehensive immune response, Pritam et al. expanded the design concept to B-cell epitopes. They designed 12 candidate vaccines MPOXVs (MPOXV1-MPOXV12) by linking different B cell epitopes into a single construct (Fig. 13A). Molecular docking analysis showed that MPOXVs can interact with TLR-2(Fig. 13B, C) and MPOXV7 had better binding ability to TLR2 than other MPOXVs. Through immune simulation verification, all 12 vaccines can induce B cell and T cell immune responses, but the B cell response is predominant [195]. Although both T cell multiple epitope vaccine and B cell multiple epitope vaccine can induce effective immune response against MPXV, T cell multiple epitope vaccine mainly induces cellular immunity, and B cell multiple epitope vaccine mainly induces humoral immunity, and neither can induce effective humoral immunity and cellular immunity at the same time.

To integrate the advantages of the first two designs and achieve the synergistic activation of humoral immunity and cellular immunity, Jin et al. conducted a comprehensive design. They combined 9 CTL epitopes, 6 B cell epitopes and 5 HTL epitopes with suitable junctions to construct a multi-epitope vaccine (Fig. 13D). Molecular docking analysis showed that MVC and TLR2 bind stably and therefore may produce a stronger immune response when interacting. Immune simulation shows that the constructed multi epitope vaccine can induce strong

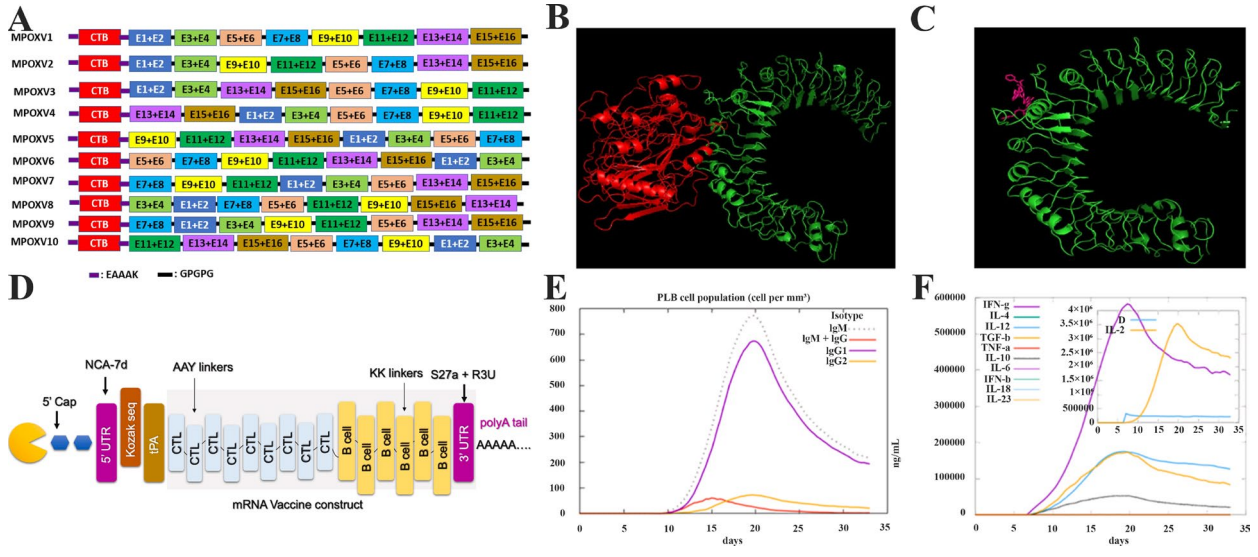

Fig. 13 Schematic diagram of multi-epitope vaccine construction, molecular docking model and immune effect. **A**) Schematic diagram of designed vaccine candidates with different combinations of epitopes. Different boxes with different colors are presenting the selected proteins and the respective epitopes are written inside the box. The plus sign between the epitopes reflects the GPGPG linker. CTB in the red box is an adjuvant CTB. **B**, **C**) Docked model of C1-TLR 2 and MPOXV1-TLR2 complex. Green, magenta, and red represent the TLR-2, control (C1), and MPOXV1. Reproduced with permission [195]. Copyright 2023, International Journal of Biological Macromolecules. **D**) Topographical organization of mRNA vaccine constructed for MPXV. The different elements of the mRNA vaccine such as 5′ capping, poly-A tail, and Kozak sequences are tagged at a particular point. **E**) Production of primary and secondary antibodies. **F**) Shows the other immune response factors required for Ag neutralization. **D**-**F**) Reproduced with permission. [197] Copyright 2023, Computers in Biology and Medicine

humoral and cellular immune responses against MPXV (Fig. 13E, F) [197]. This marks the evolution of the design of multi-epitope vaccines from a single type of immunity to a new stage of comprehensive immunity.

Multi-epitope vaccines, designed using immunoinformatic, represent a rational and potentially safer approach to vaccination by focusing on selected antigenic fragments [195]. Their designed nature allows for the avoidance of allergenic or immunosuppressive regions. However, the efficacy of these vaccines is wholly dependent on the accuracy of epitope prediction algorithms. They may also suffer from insufficient immunogenicity, requiring powerful adjuvants, and involve complex synthesis processes. Critically, all currently reported MPXV multi-epitope vaccines exist only as in silico models and conceptual designs. They remain in the earliest stage of development—the proof-of-concept stage—and urgently require validation through in vitro and in vivo studies to confirm their immunogenicity and protective efficacy.

In summary, next-generation platforms like DNA vaccines, RNA vaccines, multiple epitope vaccines, etc. have the characteristics of high stability and high immunogenicity [180, 181, 197]. However, these vaccines are still in the research or clinical trial stage and cannot provide complete Mpox protection, making vaccine development very difficult. This requires deepening research on MPXV surface antigens and developing more multivalent Mpox vaccines with effective epitopes to provide more complete protection.

Nanoparticle-based antigen-carrier vaccine

Designing a vaccine from an antigenic carrier can give the vaccine good stability and immune efficacy. For example, Aquafex aeolicus lumazine synthase (AaLS) is a synthesizing enzyme protein cage nanoparticle separated from Aquafex aeolicus [199], It self-assembles into an internal hollow icosahedral structure formed by 60 polymers, with a T (triangulation number) of 1, an inner diameter of 9 nm and an outer diameter of 15 nm. It has excellent heat resistance, with a melting point as high as 119.9° C. Additionally, considering its spatial structure characteristics and transportation and storage functions, it can be used for the delivery of proteins or drugs. [200, 201] Furthermore, Spycatcher-SpyTag is a covalently binding system that binds steadily and selectively in simple conditions and is expressed in a wide range of host cells.

Distinct from RNA, DNA and multi-epitope vaccines, nanoparticle-based antigen vector vaccines offer a unique technical approach: they efficiently present antigens by simulating the spatial structure of viruses to enhance immune recognition. Chen et al. constructed vectors expressing the recombinant proteins L1, A29 and A33 of MPXV [29], The antigen was loaded on AaLS by SpyCatcher-SpyTag covalent coupling system (Fig. 14A). Ultimately, uniform nanoparticles of approximately 30 nm were formed, indicating the successful construction of the nano-vaccine. The binding antigen has effective thermal stability and stronger immunogenicity. Compared with the corresponding monomers, the mice were stimulated to reach the antibody peak with less immunization times. One-time immunization of nano-vaccine can induce high-intensity antibody response, and two-time immunization can induce high titer of antibody for 2 months. In vitro virus neutralization experiment indicated that the candidate nano vaccines induced significantly higher levels of neutralizing antibodies than the singletons (Fig. 14B,C). Thermal stability tests showed that the nano vaccine stayed basically invariant after one week of storage at 37 °C, but only partially degraded at 60 ℃, showing high thermal stability. Compared with traditional platforms, the strong immunogenicity and excellent thermal stability demonstrated by nano-vaccines offer a new solution to address the limited immune effect of traditional vaccines and their reliance on cold chain transportation.

Nanoparticle-based antigen carriers offer a powerful strategy to enhance vaccine immunogenicity, primarily by presenting antigens in a highly ordered, virus-like presentation and by improving overall stability.[29] This platform can elicit stronger and potentially longer-lasting immune responses compared to conventional soluble antigens.

However, the clinical translation of nano-vaccines faces significant hurdles. Biocompatibility and biodegradability must be rigorously established for any new nanomaterial scaffold [118, 202]. The design must also be precisely tuned to modulate the desired immune response type (e.g., humoral vs. cellular) while minimizing potential toxicity. Furthermore, critical challenges remain in manufacturing and scalability, including production costs and ensuring long-term vaccine stability (shelf-life). Other factors, such as the in vivo interaction of nanoparticles with other biological substances (e.g., protein corona formation) and the optimization of administration routes, must also be comprehensively studied before widespread clinical adoption can be realized.

Discussion and conclusion

The recent global spread of the Mpox epidemic has catalyzed a transformative shift in the landscape of viral control, driven by profound innovations across diagnostics, therapeutics, and vaccinology [203]. In diagnostics, the paradigm is decisively shifting from centralized, laboratory-based PCR to rapid, portable, and highly sensitive POCT. This is exemplified by the integration of isothermal amplification (e.g., LAMP, RPA) with CRISPR-Cas systems and the development of novel nanomaterial-based biosensors. In therapeutics, the focus is evolving

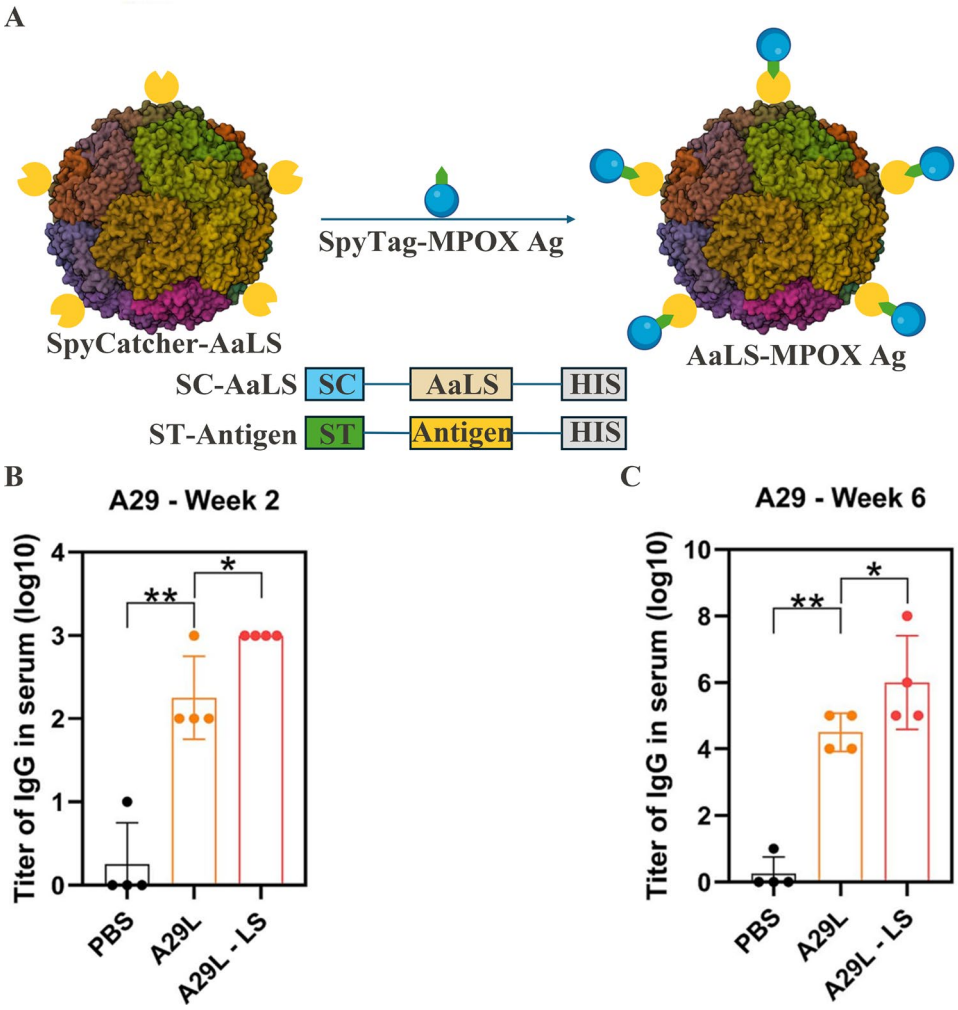

Fig. 14 Schematic diagram and its resultant humoral, and cellular immunity of multi-epitope DNA vaccine construction and techniques to enhance the efficacy of the vaccine. **A**) Schematic diagram of antigen-AaLS conjugation; **B**, **C**) The titers of anti-A29 in Week 2 and Week. Reproduced with permission [29]. Copyright 2024, Vaccine

from repurposed broad-spectrum antivirals to highly specific targeting strategies. These include monoclonal antibody cocktails, CRISPR-based viral genome editing, and "theranostic" AIEgen-based nanoplatforms that synergistically combine targeted killing with imaging. In prevention, next-generation vaccines—particularly multivalent mRNA/DNA platforms and rationally designed nanoparticle-based antigen carriers—show immense promise for inducing robust, durable immunity, often with the added benefit of improved thermal stability.

The most promising future avenues lie at the convergence of these fields: developing multiplexed CRISPR diagnostics, advancing "theranostic" nanoplatforms, and designing universal Orthopoxvirus vaccine candidates. However, critical challenges remain. These include the need to overcome viral mutagenesis that threatens diagnostic and therapeutic efficacy, conduct comprehensive safety and toxicology evaluations of novel nanomaterials and gene-editing tools, determine the optimal antigen combinations for vaccines, and address the cost and scalability of these advanced solutions for global equity. Future research must therefore be interdisciplinary, bridging foundational virology with translational engineering, to convert these promising technological paradigms into practical, accessible, and effective tools for definitively curbing the Mpox threat.

Challenges and outlook

While the advancement of novel strategies for Mpox management is highly promising, significant translational hurdles remain. Future research must pivot towards the following forward-looking directions to successfully translate these technologies from bench to bedside and construct a resilient defense system against MPXV and future Orthopoxvirus threats.

1.The high mutational rate of MPXV underscores the need for adaptive diagnostic platforms. Future efforts should prioritize multiplexed assays capable of simultaneously detecting emerging MPXV variants and other common vesiculopustular pathogens to ensure accurate differential diagnosis. Integrating CRISPR-based detection with microfluidic or biosensor technologies [204], coupled with machine learning algorithms for signal interpretation, could create "smart" diagnostic systems with enhanced robustness against viral evolution.

2. The sensitivity limitations of traditional AuNPs highlight a clear opportunity for innovation in probe design. Research should focus on synthesizing cost-effective, robust nanomaterials with superior optical properties, such as engineered quantum dots (QDs) or upconversion nanoparticles (UCNPs). Furthermore, integrating Surface-Enhanced Raman Scattering (SERS) with artificial intelligence for automated spectral analysis promises to enable ultra-sensitive, real-time detection in point-of-care settings. [205].

3. The nascent field of nanotherapy for Mpox must evolve beyond simple photothermal and photodynamic killing. A promising avenue lies in designing "smart" nanocarriers that are biodegradable, target-specific, and capable of co-delivering broad-spectrum antiviral agents (e.g., cidofovir analogs) and immunomodulators.[206] This dual-action approach—simultaneously eliminating the virus and regulating the host immune response—could overcome drug resistance and substantially improve clinical outcomes in severe cases.

4. The future of Mpox vaccination lies in rational, structure-based design. Leveraging structural biology to identify conserved, critical neutralizing epitopes across Orthopoxvirus proteins can guide the development of multivalent vaccines that elicit broad and robust protection.[207] Nanoplatforms, such as self-assembling protein nanoparticles (SAPNs), can be engineered to display these selected antigens in a highly repetitive, virus-like presentation—a strategy known to elicit stronger and more durable immune responses than conventional formulations.

5. To enhance immunogenicity and ensure global accessibility, novel delivery methods warrant greater exploration. Research into dissolvable microneedle patches for intradermal delivery,[208] inhalable formulations targeting mucosal immunity,[209] or single-dose, slow-release systems[210] could revolutionize vaccination campaigns. These platforms would simplify logistics, improve patient compliance, and potentially elicit more comprehensive immune protection.

In summary, overcoming the current limitations in Mpox control will require a concerted, interdisciplinary effort. The convergence of nanotechnology, synthetic biology, structural immunology, and computational sciences presents an unprecedented opportunity. This synergy will be essential to develop disruptive solutions that not only curb the current Mpox threat but also enhance our preparedness for future Orthopoxvirus emergencies.

Acknowledgements
This research was funded by the National Key Research and Development Program (2024YFC2311503), National Natural Science Foundation of China (82272248, 82322042, 82500159, 22565018), Yunnan Revitalization Talent Support Program, Open Competition Mechanism to Select the Best Candidates for Key Research Projects of Ningxia Medical University (XJKF230124).

Author contribution
W.G. and L wrote the main manuscript text and Z prepared Figs. 1–14. All authors reviewed the manuscript.

Funding
National Key Research and Development Program of China, 2024YFC23115032021YFC2302200, National Natural Science Foundation of China, 82272248, 82322042, 82500159, 22565018, Yunnan Revitalization Talent Support Program, Open Competition Mechanism to Select the Best Candidates for Key Research Projects of Ningxia Medical University, XJKF230124.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval
The review does not involve human subjects or animals.

Competing interests
The authors declare no competing interests.

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Received: 19 July 2025 / Accepted: 17 November 2025
Published online: 08 March 2026

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