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Biosafety materials: Ushering in a new era of infectious disease diagnosis and treatment with the CRISPR/Cas system



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ABSTRACT

Despite multiple virus outbreaks over the past decade, including the devastating coronavirus disease 2019 (COVID-19) pandemic, the lack of accurate and timely diagnosis and treatment technologies has wreaked havoc on global biosecurity. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas) system has the potential to address these critical needs for tackling infectious diseases to detect viral nucleic acids and inhibit viral replication. This review summarizes how the CRISPR/Cas system is being utilized for the treatment and diagnosis of infectious diseases with the help of biosafety materials and highlights the design principle and *in vivo* and *in vitro* efficacy of advanced biosafety materials used to deal with virus attacks.

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1. Introduction

Biosafety materials refer to materials used to prevent and control biosafety problems. With the global outbreak of the coronavirus disease 2019 (COVID-19), biosafety materials are garnering much attention [1]. The concept of biosafety refers to all security problems caused by biological risk factors, including viruses, bacteria, alien species invasion, and other events. Therefore, biosafety materials must have the ability to prevent and control biological threats [2]. These materials proved to be highly efficient in a biosafety crisis by preserving the human body and other species from harm by establishing a solid biosafety line of defense for organisms subject to a novel invasion and mediating a quick response in a short amount of time [1]. Knowing

the large range of possibilities granted by biosafety materials, the biosafety materials industry is foreseen to rapidly catch up with the shortage of biosafety materials and lack of protective equipment, which will significantly strengthen and improve China's defense against biological threats. However, the frequent occurrence of natural disasters nowadays causes various novel viruses and microorganisms to slowly "wake up." These constitute unknown biological threats that are often the cause of biosafety incidents. Biosafety materials can be used not only as a core element in the diagnosis and treatment of diseases but also as an "umbrella" for other methods of diagnosis and treatment. For example, they can be used as a safe delivery vehicle for drugs/reagents [3,4], including chemical drugs, genetic drugs, and others. Therefore, biosafety materials have broad application prospects in the detection and treatment of diseases.

From 2019 until today, the world has directly faced the threat of a global pandemic [5,6]. Since its first appearance, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has exhibited characteristics that made it extremely difficult to control. Among them are its rapid human–human transmission and the varying degrees of variations in the transmission process [7]. These characteristics made

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SARS-CoV-2 a common global nightmare that inflicted an immeasurable impact on the global economy and society [6,8]. Such a severe situation compels the scientific community to develop detection techniques that have a range of effective parameters, such as efficiency, suitability, and low cost [9]. Furthermore, the technology must be adaptable to virus mutations, with a potential for redesign when necessary. In line with this need, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas) technology, which can be used to identify viral genome sequences, shows great potential for immediate diagnostics and editable sequence-based antiviral therapies.

Both viral and nonviral vectors have been studied for the delivery of CRISPR/Cas systems. However, owing to the problems of gene mutations, insertion mutations, and immunogenicity in virus-mediated CRISPR/Cas delivery, nonviral vectors, particularly those based on biosafety materials, are urgently needed. Compared with viral vectors, biosafety material-based nonviral vectors have more advantages, such as large gene packaging capacity, safety, and targeted delivery [10]. Therefore, the limitation of the clinical application of the CRISPR/Cas genome-editing system can be solved by using biosafety materials.

This topical review highlights recent advances in the *in vivo* delivery of the CRISPR/Cas system using various biosafety materials, including lipids, polymers, peptides, inorganic materials, and extracellular vesicles. The various CRISPR/Cas detection systems are also discussed in detail.

2. Typical CRISPR/Cas systems for infectious disease diagnosis and treatment

Reverse transcription polymerase chain reaction is currently the most widely used standard detection method. However, this technique generally needs to be performed in well-equipped laboratories that meet biosafety standards and requires trained operators/personnel and specific experimental reagents and instruments. These requirements are not conducive for the timely detection and classification of suspected cases and efficient result analysis, which may result in biosafety problems. Combining the just-in-time detection features of the CRISPR/Cas system with biosafety materials enables on-site sampling and testing. Herein, we summarize the latest detection techniques and methods involved in the detection of pathogenic microorganisms and discuss the specific application of this technology to provide relevant references related to the latest technology that can be used as an alternative in areas where appropriate detection is hindered by the lack of well-equipped laboratories.

2.1. CRISPR/Cas9

The application of biosafety materials in combination with the CRISPR/Cas system can greatly improve test portability and readability. Cas9 cleavage can be combined with nucleic acid amplification for detecting specific nucleic acid sequences, which can be used to genotype pathogens and distinguish single-nucleotide polymorphisms (SNPs) [10,11]. Zika virus (ZIKV), spread by the bite of an infected *Aedes aegypti* mosquito, emerged in Southeast Asia and several Pacific islands before spreading throughout Latin America and the Caribbean (Fig. 1 A) [12]. Globally, quantitative real-time polymerase chain reaction (qPCR) is the gold standard for detecting viral RNA [13]. However, qPCR requires well-equipped laboratories and trained technicians for sample preparation. In addition, the required reagents must be refrigerated and the equipment used is expensive and energy-intensive [14]. In contrast, some simple devices, such as lateral flow test devices that use antibodies to directly detect viral antigens, have

low sensitivity and may exhibit the problems of crossreactivity with locally prevalent dengue viruses [15].

Therefore, detection methods that can quickly and accurately distinguish ZIKV are urgently needed. Pardee et al. successfully used nucleic acid sequence-based amplification and Cas9 effector for ZIKV detection and genotyping, achieving remarkable results [12]. A single guide RNA (sgRNA) with a strain-specific proto-spacer adjacent motif sequence was designed to cover SNPs present only in the American ZIKV genome but not in the African ZIKV genome. In the presence of sgRNA, Cas9 could only lyse the amplified products of the American ZIKV genome (Fig. 1 B). To increase the practicability of this system, primers for isothermal RNA amplification and a toehold switch-based RNA sensor were designed *in silico*. In less than a day, ZIKV sensors can be embedded in paper and freeze-dried with cell-free transcription and translation systems for field deployment as a stable diagnostic tool. Detection of the appropriate trigger RNA was indicated by a change in the color of the paper plate from yellow to purple in approximately 3 h. The whole process, which took less than 7 days from preparation to detection, can be used to effectively handle numerous detection needs created by outbreaks. Further, the storage time of > 1 year and storage temperature of 20 °C greatly reduced the transportation and storage costs, thereby satisfying the needs of low-cost testing (Fig. 1 C).

The combination of the lateral flow test and CRISPR/Cas can compensate for the shortcomings of the lateral flow test and improve the detection accuracy [16]. In addition to communicable diseases, similar tests can be used for foodborne diseases. A lateral flow test strip combined with Cas9 nickase-triggered isothermal DNA amplification was previously used in food quality surveillance [17].

2.2. CRISPR/Cas13

Cas13a is an RNA-guided RNA enzyme with CRISPR RNA (crRNA)-guided single-stranded RNA (ssRNA) cleavage activity [18,19]. In 2016, Cas13a was found to have target RNA triggering “side-chain cleavage” activity [20]. After further exploring this activity, Zhang et al. developed the first comprehensive and adaptable CRISPR/Cas13-based nucleic acid detection system called specific high-sensitivity enzymatic reporter unlocked (SHERLOCK) [21]. This method can achieve aM (10^{-18} mol/L) sensitivity for DNA and RNA targets with single-base resolution and has also been used to detect SARS-CoV-2 and other viruses [5].

The SHERLOCK-based nucleic acid detection system has a wide range of applications in the detection of infectious diseases that affect biosecurity. To protect human biosecurity, Cameron et al. used the SHERLOCK platform to detect ZIKV and dengue virus at concentrations as low as 1 copy/ μ L [22]. This system distinguished four dengue virus serotypes and region-specific strains of ZIKV from the 2015–2016 pandemic, proving to be a sensitive, specific, and rapidly field-deployable diagnostic tool for infectious diseases worldwide [22]. Similarly, canine parvovirus type 2 (CPV-2) causes a canine disease with high mortality. Haroon et al. used the SHERLOCK molecular detection system for specific high-sensitivity enzyme reporter genes to assess the sensitivity of this system for detecting CPV-2 DNA [23]. The nanosystem can detect 100 aM CPV-2 DNA in 30 min, which is time-efficient, effortless, and does not involve the use of complex instruments.

Subsequently, SHERLOCKv2, an update of SHERLOCK, was developed. Compared with SHERLOCK, SHERLOCKv2 has 3.5-fold better sensitivity owing to the combination of Cas13a with Csm6 [24]. Csm6 is an auxiliary type III CRISPR-triggered nuclease capable of binding its reporting signal to Cas13a for signal enhancement [14]. A portable paper lateral flow test strip was prepared by combining the FAM biotin reporter gene with anti-FAM antibody and gold nanoparticles [24].

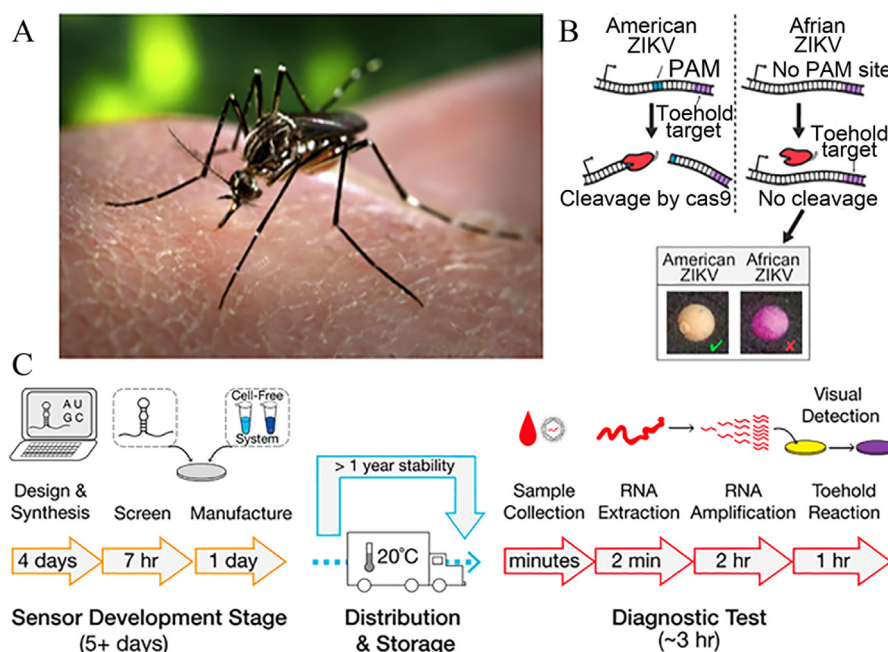


Fig. 1. Application of CRISPR/Cas9 in the detection of Zika virus. A) Zika virus is transmitted through the bite of an infected *Aedes aegypti* mosquito [15], Copyright 2016 Elsevier. B) Via CRISPR/Cas9 can distinguish between American and African Zika virus types [12]. C) Workflow for rapid prototyping of paper-based biomolecular sensors for portable and low-cost diagnostics [12], Copyright 2016 Elsevier.

2.3. CRISPR/Cas12

The class II V-A Cas12 (formerly known as Cpf1) effectors have lateral (or *trans*-cutting) activity [25–27]. However, unlike the Cas13 effector, the Cas12a effector targets DNA and transects side-branch single-stranded DNA (ssDNA), activating the DNA enzyme activity of Cas12a while cleaving the targeted double-stranded DNA (dsDNA). Cas12a cleaves ssDNA in a nonspecific manner [27]. In 2017, the *trans*-cutting activity of Cas12a and its application in nucleic acid detection (1-h low-cost multipurpose highly efficient system or HOLMES) was first reported in China, and a patent for the same was granted [28]. HOLMES can be used to detect DNA/RNA viruses and distinguish between viral genotypes and human SNPs from cell lines or clinical samples within 1 h with aM sensitivity. Moreover, Doudna's team at the University of California developed another new Cas12a-based technique, called DNA endonuclease-targeted CRISPR trans reporter or DETECTR, that could detect Human Papilloma Virus (HPV)-16 infection with 100% accuracy [26]. Based on this system, Kean's team developed an engineered AsCas12a enzyme for the diagnostic analysis of SARS-CoV-2, which is sensitive to viral genome mutations and temperature [29].

In response to the unprecedented need for rapid diagnostics in the COVID-19 pandemic, Park's team developed the first digital CRISPR/Cas-assisted assay, called digitization-enhanced CRISPR/Cas-assisted one-pot virus detection (deCOViD) [30]. More important than micro-liter volume analysis, deCOViD uses analytical digitization to increase RNA concentrations in the subnanoliter digital reaction wells and facilitates rapid amplification, resulting in faster detection times at lower target concentrations as well as higher signal transfer rates, wider dynamic range, and better sensitivity.

The class II V-B CRISPR/Cas12b has also shown the same DNA *trans*-cleavage activity. In 2019, Li et al. created an improved version of HOLMES (called HOLMESv2) based on Cas12b [31]. HOLMESv2 can target both ssDNA and dsDNA. It is one of the simplest CRISPR/Cas biosensing systems available for DNA detection.

3. Application of biosafety materials in infectious disease diagnosis and treatment

3.1. Lipid materials

Lipid nanoparticles (LNPs) are one of the most effective nucleic acid delivery systems [32,33]. LNPs have been widely used in the delivery of nucleic acids, including DNA, siRNA, and mRNA, due to their excellent transfection ability and low toxicity [3]. The key lipids of LNPs are usually ionizable lipids that possess hydrophilic heads and hydrophobic tails, allowing LNPs to spontaneously assemble into nanoparticles in an aqueous environment and disassemble and release drugs in acidic environments.

Suzuki et al. reported an LNP-based CRISPR/Cas ribonucleoprotein (RNP) delivery nanosystem, which was synthesized using a microfluidic device and completely prevented DNA cleavage activity and Cas enzyme aggregation. This system was used in therapeutic editing for HBV-infected hepatitis. Single-stranded oligonucleotides (ssON) were added to the RNPs to obtain more negative charges. Subsequently, RNP-loaded LNPs were obtained by mixing the RNP-ssON complex in the form of an aqueous solution with a mixture of lipids in ethyl alcohol (PEG lipids, cholesterol, phospholipids, and pH-sensitive cationic lipids) in a microfluidic device through electrostatic action (Fig. 2). Compared with adeno-associated virus type 2 (AAV2), LNPs showed better gene editing efficiency in Hepatitis B virus (HBV)-infected HepG2 cells and significantly inhibited the replication of HBV DNA and covalently closed circular DNA (cccDNA) [34].

Li et al. developed lipid-like nanoparticles (TT3) to deliver the Cas9 mRNA/sgRNA complex. The Cas9 mRNA targeting the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene was first injected to achieve significant gene knockout. This blocked HBV infection and showed high editing performance and anti-HBV effect [35,36].

In a recent study, Gao et al. used cationic lipids to deliver Cas9 RNPs in which sgRNA was designed to target transmembrane channel gene family 1 (Tmc1) genes to treat autosomal dominant hearing loss in hair

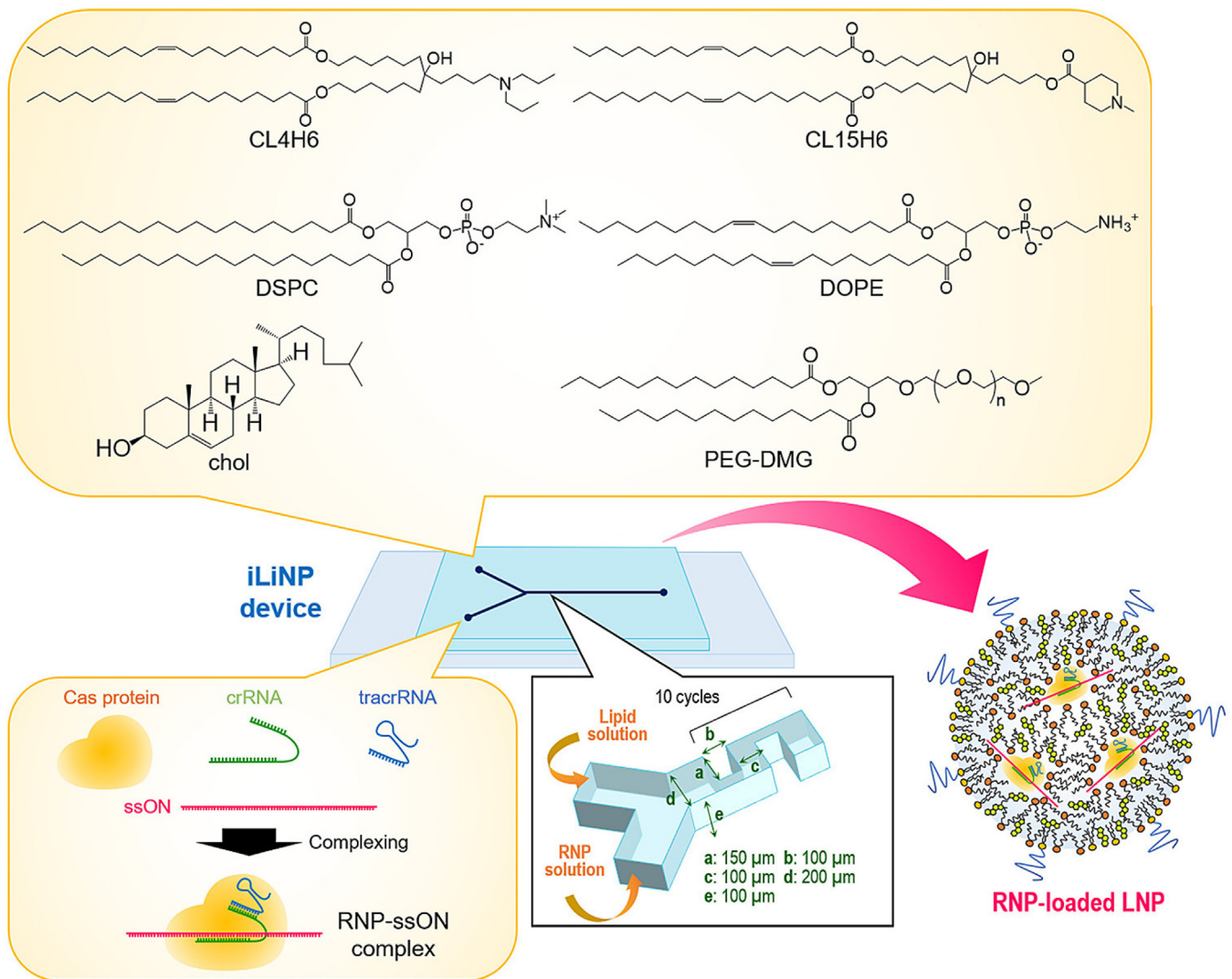


Fig. 2. Schematic of the synthesis of RNP-loaded LNPs using a microfluidic device [34], Copyright 2020 Elsevier.

cells. Nanocomposites were directly injected into the cochlea of newborn *Tmc1^{Bth/+}* mice. The results revealed that the auditory brainstem response threshold was lower and the auditory shock response level was better in the experimental group than in the control group [37].

In addition to delivering RNPs, LNPs can deliver plasmids and mRNA. Commercial LTX lipids were used to deliver streptococcus pyogenes Cas9 (SpCas9)-sgRNAs plasmids and subsequently inactivate HBV by targeting multiple HBV domains *in vitro* [38]. Additionally, researchers make use of the advantages of nonviral vectors by combining viral vectors with nonviral vectors to solve the problem of insufficient packaging capacity of viral vectors. Yin et al. developed a combined system comprising LNPs and adeno-associated viruses (AAVs), in which Cas9 mRNA was encapsulated in LNPs and homology directed repair/sgRNA templates were loaded in AAVs. After treating Fah knockout mice with this combined delivery system for 30 days, the markers of liver injury were significantly reduced and the body weight was stable in the experimental group, indicating that CRISPR/Cas9-based gene therapy has great potential in the treatment of genetic liver diseases, such as metabolic liver disease and haemophilia [39].

3.2. Polymers

The excessive use of antibiotics plays a major role in the emergence and transmission of multidrug-resistant bacteria [40]. Using the

CRISPR/Cas system to specifically target bacterial pathogens for gene editing reduces microbial growth levels. Cas9 proteins were covalently modified using cationic polymers and then mixed with targeted antibiotic-resistant sgRNA to form small nanocomplexes (Fig. 3 A). Studies have shown that polymer-derived nanocomposites can be successfully introduced into methicillin-resistant *Staphylococcus aureus* to effectively edit the bacterial genome, thereby decreasing the growth of this organism [41].

HPV infection is the main cause of cervical cancer, and the oncogene *E7* plays an important role in HPV carcinogenesis. Zhu et al. developed HPV16 *E7* targeted nanoparticles composed of poly b-amino esters and CRISPR/short hairpin RNA [42]. Their results showed that the nanoparticles successfully reduced the expression of HPV16 *E7*, inhibited the growth of cervical cancer cells and xenograft tumors in nude mice, and reversed the malignant phenotype of cervical epithelium in HPV16 transgenic mice.

The inflammatory corpuscle nod like receptor protein 3 (NLRP3) is a therapeutic target for many inflammatory diseases. A cationic lipid-assisted nanoparticle (CLAN) library with different surface charges and surface PEG densities was prepared by adjusting the amount of *N,N*-Bis(2-hydroxyethyl)-*N*-methyl-*N*-(2-cholesteryoxycarbonyl-aminoethyl) ammonium bromide (BHEM-Chol) or polymers (PEG_{5K}-b-PLGA_{11K} and PLGA_{11K}) (Fig. 3 B). Subsequently, macrophage uptake of CLAN_{Cy5-siRNA} was evaluated by fluorescence activated cell

sorting (FACS) to encapsulate Cas9-enhanced green fluorescent protein (EGFP) or Cas9 mRNA and sgRNA. Based on efficient EGFP expression and green fluorescent protein (GFP) knockout, the generation of an optimized CLAN for macrophage gene editing was confirmed. After intravenous injection, an optimized CLAN was used to deliver Cas9 mRNA/gNLRP3 into macrophages, blocking the NLRP3 gene and alleviating inflammatory diseases. Additionally, insulin sensitivity and adipose inflammation of high-fat-diet-induced type 2 diabetes can be improved with Cas9 mRNA/gNLRP3 treatment [43]. CLAN can also be used to deliver plasmids for genome editing *in vivo*. Wang et al. used CLAN to deliver the pX330 plasmid (pCas9/gNE) expressing Cas9 and gRNA targeting neutrophil elastase (NE) gene into mice and successfully knocked out the NE gene in neutrophils [44].

3.3. Peptides

One of the characteristics of Alzheimer's disease is the accumulation of amyloid β peptides. Considering that the formation of amyloid β peptides is regulated by beta-secretase (Bace1), targeting Bace1 is a potential strategy for treating Alzheimer's disease. Krishnamurthy et al. combined the amphiphilic R7L10 peptide with the Cas9/gRNA RNP complex through electrostatic interaction for neuronal gene editing in Alzheimer's disease [45].

Krishnamurthy et al. developed an amphiphilic shuttle peptide platform for delivering Cas9-sgRNA and Cas12-crRNA to airway epithelial cells, which is expected to contribute to the prevention of respiratory infections [46]. The amphiphilic shuttle peptide can enhance the uptake of the complex by respiratory epithelial cells, providing a potential pathway for the treatment of airway epithelial cells.

Zhang et al. developed a self-assembling peptide coating on nanofibers, which can guide the local transmission of the CRISPR/Cas9 system and promote nerve regeneration. Notably, they indicated that SAP coatings did not significantly alter the topography of the aligned fibers. First, negatively charged amphiphilic SAP⁻ (FEFEFEFE, self-assembling peptide) were coated onto polycaprolactone (PCL) nanofibers via strong hydrophobic interaction. Subsequently, plasmid DNA/PEIpro (pDNA/PEIpro) complex and SAP⁺-RGD (FKFKFKFKGGGRGD SP, self-assembling peptide) were adsorbed by electrostatic interaction, inducing the expression of glial cell-derived neurotrophic factor (GDNF) and production of GDNF in mammalian cells (Fig. 4). The secreted GDNF maintained its biological activity and promoted the sudden growth of the meridian, which was twice as long as that in the untreated group [47].

Gene deletion or editing in adipose tissue to enhance energy consumption, fatty acid oxidation, and bioactive factor secretion (through browning) is a potential therapeutic strategy for alleviating metabolic diseases. Shen et al. designed an amphipathic α -helical peptide called the Endo-Porter (EP). They complexed Cas9 RNP with EP peptide to form a nanocomplex (CriPs). CriPs disrupted the nuclear corepressor of catabolism, nuclear receptor-interacting protein 1, and enhanced adipocytes browning, suggesting a promising treatment for metabolic diseases [48].

3.4. Inorganic materials

Conditional responsive gene editing has shown advantages in disease treatment in recent years. Ju et al. developed pH-dependent self-assembling gold nanoclusters (AuNCs). The assembly of gold nanoclusters (AuNCs) and SpCas9 proteins mainly depended on the pH value. SpCas9–AuNCs exhibited better affinity for higher pH values, evidenced by their ability to be highly stable at relatively high pH values. Further, SpCas9–AuNCs were unstable at lower pH values and appeared dismantled in most cases (Fig. 5 A). SpCas9–AuNCs effectively knocked out the oncogene *E6* after the transfection of HPV18 E6 sgRNA, thereby restoring the function of the tumor suppressor protein p53 and inducing cell apoptosis [49].

External conditions that guide on-demand drug release have controllable advantages in the treatment of several diseases. Kaushik et al. demonstrated that a nanoformulation consisting of a combination of Cas9/gRNA with magneto-electric nanoparticles (MENP) crosses the blood–brain barrier noninvasively under magnetic guidance to inhibit latent HIV-1 infection in microglia (μ glia)/HIV (HC69) cells. An optimized AC magnetic field of 60 Oe at 100 kHz was applied, causing the repeated contraction and expansion of the MENP–Cas9/gRNA bond length. Subsequently, the MENP–Cas9/gRNA bonds broke down at some points. Cas9/gRNA was released from the MENP surface, thereby achieving intracellular release and HIV inhibition [50] (Fig. 5 B).

AuNPs can be easily modified by altering their charge, hydrophilicity, and functional ligands. A high level of low-density lipoprotein cholesterol (LDL-C) in the blood is a major risk factor for coronary heart disease. Zhang et al. developed an AuNP/CRISPR/Cas9 system to knock out the *Pcsk9* gene and reduce the levels of plasma LDL-C. First, gold nanoclusters (GNCs) were modified by TAT (cell-penetrating peptide) peptides to form nuclear-targeted clusters (TAT-GNCs). TAT-GNCs, nuclear localization signal (NLS)-labeled Cas9 protein, and sgPcsk9 were mixed to form an anion complex (GCP). GCP was further encapsulated in the galactose-modified lipid layer to form a triple targeting system [galactose targeting asialoglycoprotein receptor (ASGPR), TAT peptide, and NLS targeting the nucleus], in which Cas9 and sgPcsk9 can be delivered to the nuclei of hepatocytes to achieve gene editing and reduced plasma LDL-C [51].

In vitro CRISPR/Cas gene editing of hematopoietic stem cells and progenitor cells (HSPCs) has opened up potential therapeutic models for several diseases. The AuNP CRISPR/Cas system was developed for genome editing in HSPCs. Guide RNAs (crRNAs) with an oligoethylene glycol spacer arm and a thiol group were conjugated to the surface of the gold cores. Cas9 or Cpf1 was connected to crRNA by natural affinity, following which the RNP-loaded AuNPs were coated with PEI. Subsequently, the single-stranded DNA (ssDNA) donor was adsorbed onto the surface by electrostatic interaction. The experimental results showed that AuNPs can effectively transfect Cas9 or Cpf1 RNP/ssDNA into HSPCs for *in vitro* genome editing [52].

Lee et al. designed a nanosystem composed of gold nanoparticles, Cas9 RNP, donor DNA, and the disruptive endosomal polymer poly(N-(N-(2-aminoethyl)-2-aminoethyl) aspartamide) (PAsp(DET)) to perform Cas9-initiated homology directed repair. Once CRISPR-Gold entered the cytoplasm, glutathione released DNA from the gold core of CRISPR-Gold, resulting in the rapid release of Cas9 RNPs and donor DNA. Consequently, successful gene editing and homology directed repair were successfully demonstrated in human embryonic stem cells, induced pluripotent stem cells, and mouse models of Duchenne muscular dystrophy [53].

3.5. Extracellular vesicles

Extracellular vesicles, including exosomes and microvesicles, are biological nanovesicles containing proteins, lipids, and nucleic acids that act as natural delivery vehicles [54–56]. They are released by most cells into the extracellular space and culture media and play an important role in intercellular signaling [57]. “Gesicle” is a microvesicle produced by the overexpression of vesicular stomatitis virus G glycoprotein and has been designed as a delivery vehicle for Cas9 RNP [58]. In the study by Lee et al., Cas9 RNP was transported into microglia, carrying pre-HIV virus through “Gesicles.” They found that the rapid delivery of Cas9 RNP vesicles targeting the HIV long terminal repeat sequence resulted in mutations and copy number loss in the pre-HIV virus and decreased protein activity determined by luminescence and protein analysis [59]. Wang et al. transfected plasmids expressing Cas9 and gRNA into HuH7 cells, collected cell culture supernatant, and purified extracellular vesicles. They found that full-length gRNA and Cas9 proteins were present in the extracellular vesicles.

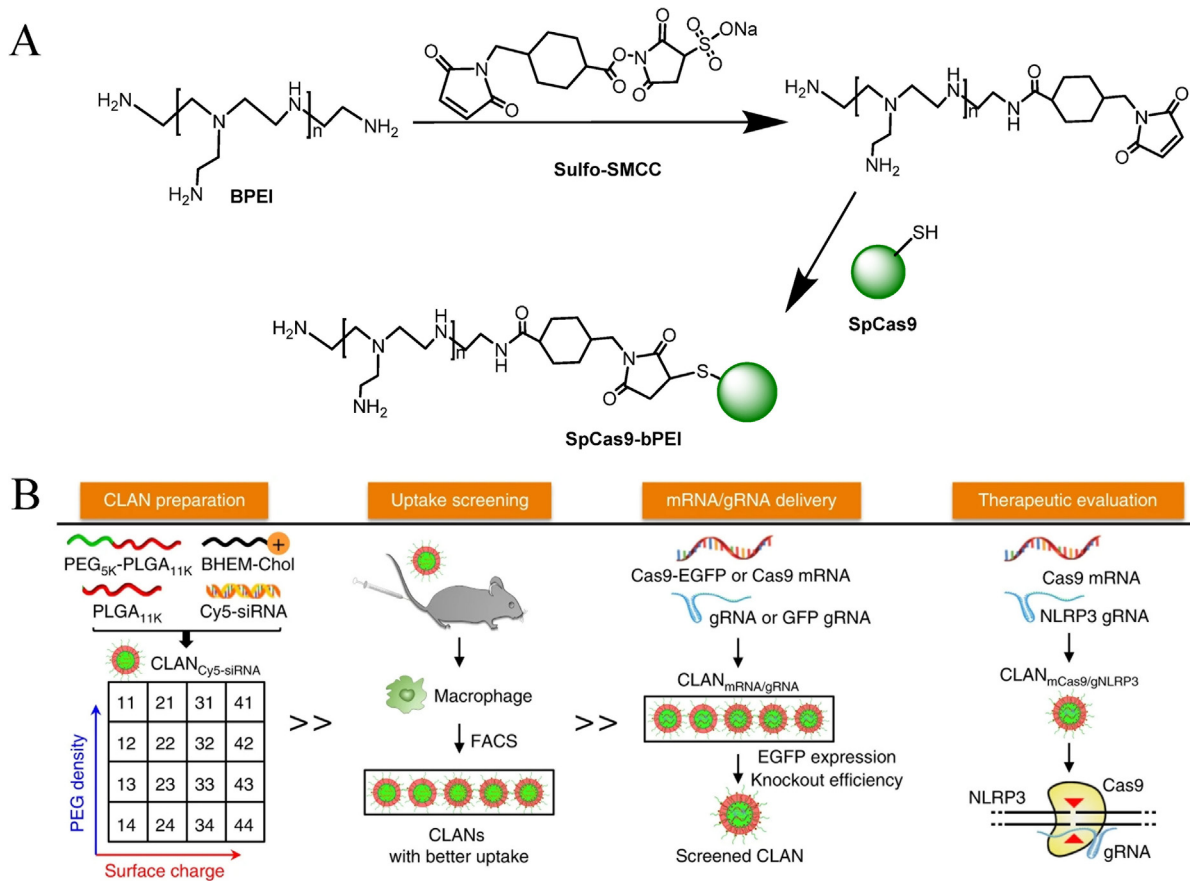
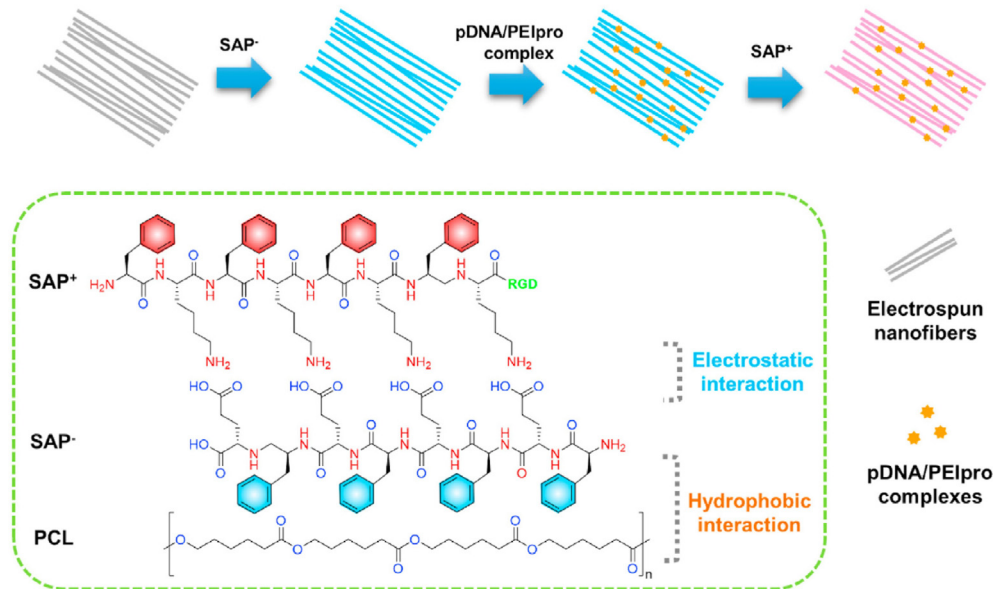


Fig. 3. Construction of polymer and protein/mRNA complexes. A) Synthesis of polymer-derivatized SpCas9 protein [41], Copyright 2017 American Chemical Society. B) CLAN preparation, uptake screening, mRNA/gRNA delivery, and therapeutic evaluation [43], Copyright 2018 the Author(s).



cles derived from HuH7 cells that were previously transfected with CRISPR/Cas9-expressing plasmids. The HBV-expressing and HBV-specific CRISPR/Cas9 plasmids were then transfected into HuH7 cells for coculture. Intercellular gene editing was achieved by destroying the HBV genome in the surrounding cells [60].

4. Challenges of biosafety materials applied in CRISPR/Cas systems

The development of biosafety materials is still in its infancy [1]. There are many challenges yet to be addressed and solved. From the

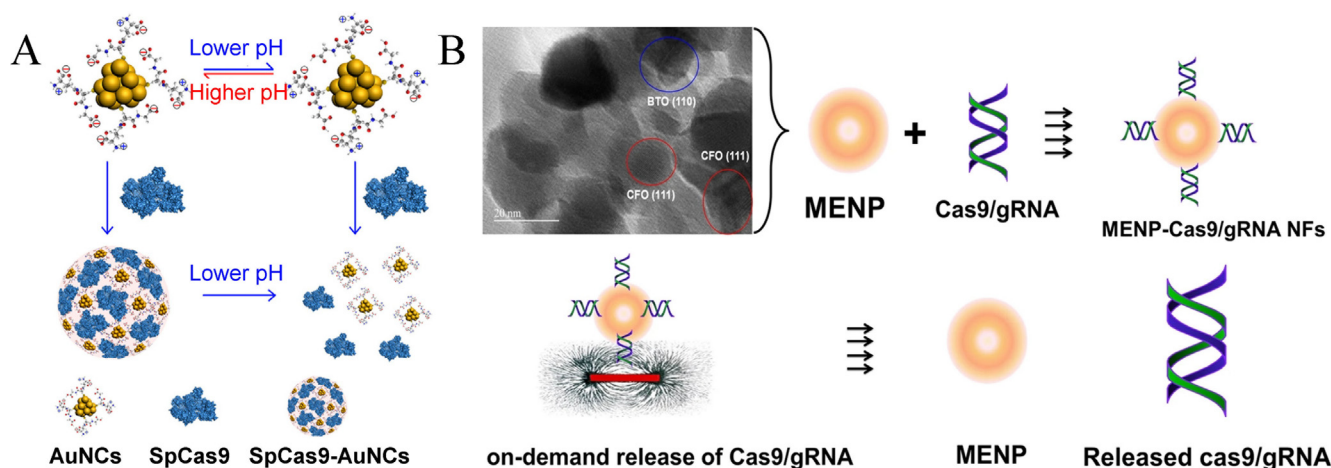


Fig. 5. Conditional gene editing triggered by inorganic RNP complexes. A) Schematic of pH-induced assembly and disassembly of SpCas9–AuNCs. SpCas9–AuNCs were highly stable at higher pH values but dismantled at lower pH values [49], Copyright 2019 American Chemical Society. B) Schematic of the preparation and release of Cas9/gRNA nanoformulation by an electromagnetic coil (60 Oe at 100 kHz for 30 min) [50], Copyright 2019 the Author(s).

perspective of applications in detection technologies, the great demand for virus detection technologies has compelled the scientific community to develop several low-cost biosafety materials. This is certainly a very challenging task, but it can substantially benefit the world, particularly the developing regions, where transmission and outbreaks of infectious diseases are more common due to poor health-care facilities. In addition to infectious diseases, the technology can also be used in daily life to detect routine pathogenic microorganisms, maintain social and economy stability. An example is the food industry, which is expected to use microorganism detection techniques to maintain food safety.

Nowadays, viral vectors, such as adenoviral vectors and AAVs, are commonly used for gene editing. However, these viral vectors are often used to deliver Cas9 and sgRNA plasmid DNA, which have limits in the delivery of Cas proteins. Compared with plasmids, Cas9 RNPs have substantial advantages in terms of higher safety and fewer off-target effects and do not cause harm to normal tissues and organs. Therefore, it is necessary to design biosafety materials using materials that not only have the ability to carry plasmid DNA, mRNA, and Cas RNPs but also do not produce immune responses. The application of biosafety materials in CRISPR/Cas systems is in the initial stage. Most applications are related to cancer treatment. There are relatively few reports on the application of biosafety materials in infectious disease therapy and diagnosis.

Gene-editing tools work at the genetic level, presenting the biggest challenge for CRISPR/Cas systems. Until now, most CRISPR/Cas research has been conducted at the cellular and test-tube levels. However, the ultimate goal of this technology is to achieve gene editing in living organisms, which is necessary to solve the specific problem. CRISPR/Cas systems may produce serious off-target effects. Pattanayak et al. proved that the off-target effects was up to 84% [61]. Recently, researchers have conducted several studies to reduce the off-target effects of CRISPR/Cas systems [62,63]. Several excellent reviews focusing on this topic have also been published [64–66]. The direct delivery of Cas9 protein and sgRNA into cells is more efficient than plasmid delivery because it eliminates the Cas9 expression step and reduces off-target cleavage. The large molecular weight of RNPs requires an appropriate delivery vector to properly carry the protein, posing challenges for the accurate design and application of biosafety materials.

5. Perspective

Despite the rapid advances in CRISPR/Cas technology over the past decade, viral vectors remain the dominant delivery vehicles. Several

nonviral vectors have emerged, including lipids, polymers, peptides, inorganic materials, and extracellular vesicles. The safety of materials has always been a concern for these carriers. As foreign substances, these materials will be recognized as antigens under certain conditions, leading to phagocytosis and clearance by immune cells, resulting in immune effects. This poses new requirements for material innovation. These materials can be endowed with various properties to evade immune recognition. They can be modified with targeted groups and ligands for immune evasion to improve specificity and immune escape [67]. For example, CD47 provides the “Don’t eat me” signal. Additionally, gene editing can be initiated by internal/external conditions, such as local microenvironmental changes and suitable light stimulation. For extracellular vesicles, which are natural carriers, a reliable way to obtain stable quality between batches is yet to be established. Among the various delivery vehicles, lipids are most widely used in CRISPR/Cas systems owing to their safety and convenience. Biosafety materials should be able to be used in the detection and treatment of diseases and exhibit good biosafety characteristics. Therefore, biosafety materials should be reasonably designed for use in the delivery of the CRISPR/Cas system gene-editing tool to enable CRISPR/Cas system to enhance ability of accurate gene regulation to step forward in clinical research and application.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

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