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Review Article

CRISPR/Cas9 systems: Delivery technologies and biomedical applications

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ABSTRACT

The emergence of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome-editing system has brought about a significant revolution in the realm of managing human diseases, establishing animal models, and so on. To fully harness the potential of this potent gene-editing tool, ensuring efficient and secure delivery to the target site is paramount. Consequently, developing effective delivery methods for the CRISPR/Cas9 system has become a critical area of research. In this review, we present a comprehensive outline of delivery strategies and discuss their biomedical applications in the CRISPR/Cas9 system. We also provide an in-depth analysis of physical, viral vector, and non-viral vector delivery strategies, including plasmid-, mRNA- and protein-based approach. In addition, we illustrate the biomedical applications of the CRISPR/Cas9 system. This review highlights the key factors affecting the delivery process and the current challenges facing the CRISPR/Cas9 system, while also delineating future directions and prospects that could inspire innovative delivery strategies. This review aims to provide new insights and ideas for advancing CRISPR/Cas9-based delivery strategies and to facilitate breakthroughs in biomedical research and therapeutic applications.

1. Introduction

Gene editing technology involves the generation of DNA double-strand breaks (DSBs) at specific sites of DNA using artificial endonucleases and then initiates the natural repair mechanisms to achieve effective targeted genome editing in various organisms and cell types [1]. The proposal that homologous recombination could be utilized for therapeutic purposes marked the inception

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of genome editing technology in 1986 [2]. Since then, with the development of artificial endonuclease, artificial endonuclease-mediated gene editing technology has gone through many stages, such as nuclease technology, zinc finger protein (ZFP) technology and transcriptional activator-like effector nuclease (TALEN) technology, but the advancement of TALEN faced substantial obstacles due to inadequate delivery efficiency, unintended off-target effects and immune responses. Recently, CRISPR/Cas9 technology emerged as a game-changer in the field of gene editing, which showed more advantages than other gene-editing technologies. CRISPR/Cas9 has demonstrated immense potential in the realm of genetic manipulation and has garnered widespread application in biomedical research.

In 1987, Ishino et al. discovered repetitive nucleotide sequences accompanied by unrelated and non-repetitive short sequences in *Escherichia coli*, marking the first identification of CRISPR [3]. This discovery sparked their curiosity. Following investigations have revealed that CRISPR/Cas functions as an adaptive immune system against viruses in prokaryotes. With the elucidation of the composition of the CRISPR/Cas system and its mechanisms in countering exogenous viral invasion, mounting evidence has supported its potential in human gene editing [4,5]. Since then, CRISPR technology has experienced rapid development.

Within the genomic CRISPR locus, there are three crucial components: the trans-activating CRISPR RNA (tracrRNA) gene, the Cas gene, and the CRISPR repeats and spacers. These components are transcribed into tracrRNA, Cas9 protein and precursor crRNA (pre-crRNA) correspondingly. When encountering foreign viruses, bacteria possess the ability to integrate specific genes from these viruses into their CRISPR/Cas9 locus, thus retaining a memory of the invading virus. In the face of subsequent viral invasions, bacteria can recognize exogenous DNA and initiate the cleavage of viral DNA as a mechanism of self-protection. This immune defense mechanism unfolds through three stages: The first stage involves incorporating the spacer sequence, derived from the external genetic material, into the CRISPR array. Part of the exogenous DNA sequence is integrated into the genome of the CRISPR system, thereby generating novel repetitive sequences. These repeats serve as the fundamental structure for the bacterium's self-specific immune response. The second stage involves the production of crRNA. Upon re-invasion of the exogenous DNA sequence, the CRISPR array undergoes a transcription process, resulting in the synthesis of pre-crRNA. The pre-crRNA is subsequently processed by the ribonuclease III (RNase III) and matures, forming a complex with tracrRNA. Then the tracrRNA pairs with a portion of crRNA to form the single guide RNA (sgRNA), which can guide the Cas9 protein for targeted cleavage. In the third stage, the Cas9 protein specifically targets and cleaves the invading nucleic acids, leading to the disruption of the DNA double strands. Subsequently, the broken strands are repaired through either the non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms, which can result in gene deletions, insertions and targeted mutations (Fig. 1). HDR utilizes donor DNA molecules as templates for repair, enabling accurate integration of target genes or correction of mutations. In contrast, NHEJ is prone to

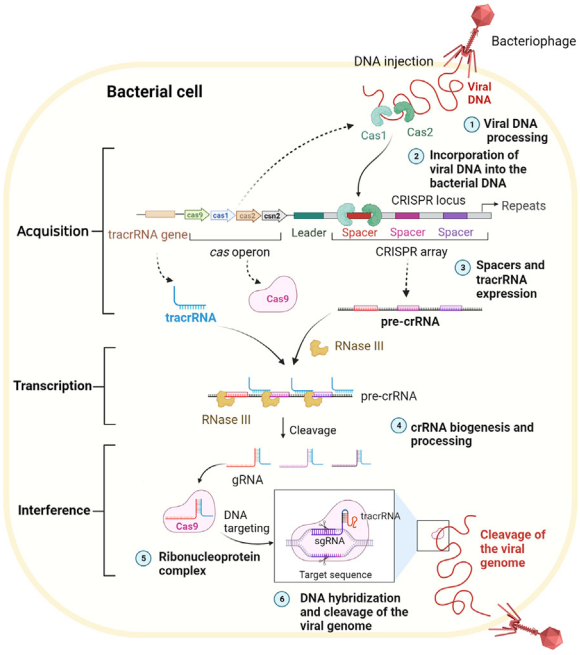

Fig. 1 – The biological mechanisms of the CRISPR/Cas9 system can be divided into several stages. During the acquisition stage, the system acquires the interval sequence of invasive nucleic acids or incorporates it into the CRISPR array. In the transcription stage, the CRISPR locus is transcribed and processed. In the interference stage, the CRISPR/Cas9 system performs targeted interference with exogenous viruses.

errors and relies on DNA ligase IV to reconnect the broken ends, often resulting in insertion or deletion mutations. NHEJ is widely employed for gene knockout purposes [6]. NHEJ represents the preferred pathway for repairing DSBs in mammalian cells and operates throughout all stages of the cell cycle. On the other hand, HDR predominantly occurs during the S/G2 phase when undamaged sister chromatids (or donor DNA) are available for repair utilization [7] (Fig. 2A).

The CRISPR/Cas9 system represents a significant departure from previously utilized gene-editing tools. It no longer simply inserts exogenous DNA into the genome and instead focuses on the elimination of faulty DNA and substitution with correct DNA. Theoretically, precise manipulation of the genome can be achieved by designing specific sgRNA. Extensive optimization of Cas9 endoribonucleases ensures efficient expression and high-fidelity efficacy across diverse organisms. Cas9 endoribonucleases have been extensively optimized to ensure efficient expression and high-fidelity efficacy across different organisms [8]. CRISPR/Cas9 gene editing facilitates a wide range of accurate modifications, including gene insertion, gene knockout, alteration of individual bases, regulation of gene transcription, and comprehensive genetic screening [9]. The applicability of CRISPR/Cas9 technology has expanded to numerous fields. It has fundamentally transformed the research on the pathological mechanisms and therapeutic strategies related to a wide range of diseases, such as Alzheimer's disease

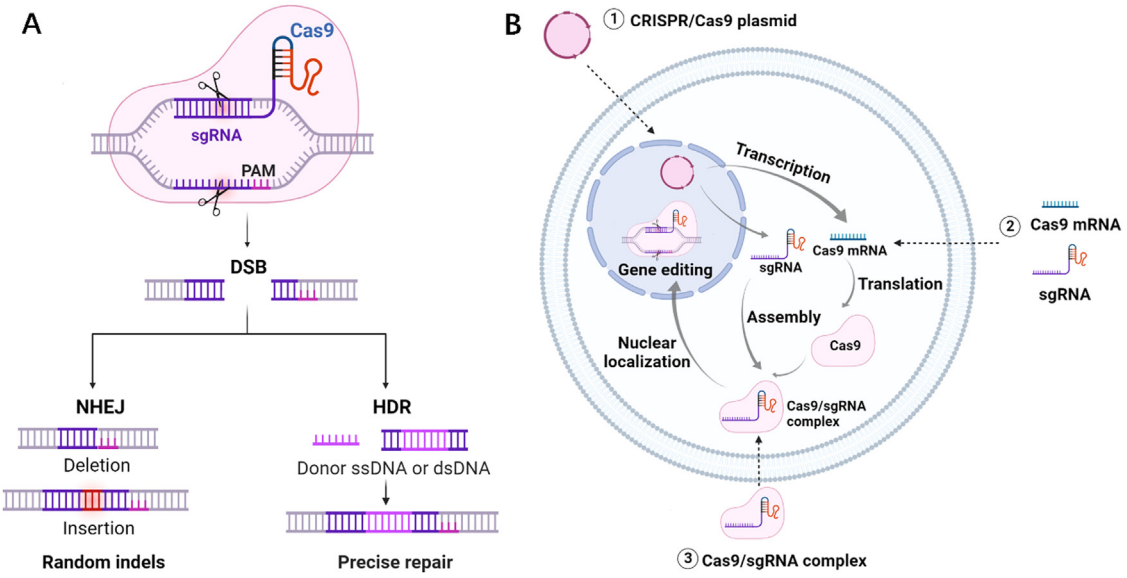

Fig. 2 – (A) Schematic diagram of the molecular mechanism of CRISPR/Cas9 system-mediated gene editing. (B) Three formats of CRISPR/Cas9 systems.

(AD) [10], AIDS [11], autoimmune diseases [12] and gene editing of plants [13] and microorganisms [14]. Although there are many reviews on the delivery of the CRISPR/Cas9 system [15,16], we present an extensive examination of the advancements achieved in various delivery systems for CRISPR/Cas9, highlighting crucial factors and difficulties linked to the delivery of CRISPR/Cas9. We suggest strategies to overcome these limitations and also offer insights into the recent progress made in employing CRISPR/Cas9 for cancer treatment and genetic disease therapy.

2. Formats and key factors of the CRISPR/Cas9 systems

The CRISPR/Cas9 system exerts its gene editing function, with both the Cas9 protein and the sgRNA being indispensable. To guarantee efficient delivery, diverse techniques can be utilized based on the specific needs of the delivery format. When choosing a delivery vector, one must carefully evaluate the molecular size and charge of the cargo. Furthermore, emphasizing the targeting accuracy and efficacy of the delivery system is of utmost importance.

2.1. *Formats of CRISPR/Cas9 systems*

The CRISPR/Cas9 systems exist in three different forms (Fig. 2B): (1) plasmids encoding both Cas9 protein and sgRNA. sgRNA and Cas9 proteins can be transcribed and expressed at the target site. This DNA format is the most stable and convenient option [17]. However, this format of delivery poses several challenges. Due to the processes of transcription and translation, there are difficulties in delivering the plasmids into the nucleus, risks of integration into the host genome, and higher potential for off-target effects. (2) sgRNA and mRNA encoding Cas9 protein. Compared to plasmids, the delivery of mRNA is simpler and safer as the subsequent translation occurs in the cytoplasm rather than the nucleus, and mRNA does not integrate with the host genome [18]. However, mRNA can degrade during its formation and delivery processes [19]. Therefore, after mRNA enters the host cells, chemical modifications should be applied to enhance its stability and ensure efficient translation [20]. (3) Cas9 protein and sgRNA can be delivered either separately or combined to form RNPs. This delivery form eliminates the risk of integration with the host genome. Additionally, the Cas9 protein degrades rapidly, reducing immunogenicity and minimizing off-target effects. Moreover, delivering RNPs exhibits better gene editing efficiency and lower off-target effects [21]. Compared to DNA and mRNA formats, RNPs are considered safer. However, scaling up the production of Cas9 protein and sgRNA remains a challenge. Table 1 summarizes the main advantages and disadvantages of the three delivery formats.

Table 1 – Three delivery forms of the CRISPR/Cas9 systems and their characteristics.

Delivery formats	Delivery payloads	Advantages	Limitations
DNA	CRISPR/Cas9 plasmid	Most simple and stable	Higher off-target effects; Low efficiency
mRNA	Cas9 mRNA and sgRNA	Faster and lower off-target effects	Vulnerable; Unstable
Protein	Cas9/sgRNA complex	Rapid and significant reduction of off-target effects, toxicity, and immune response	Difficult to obtain; Risk of permanent integration into the host

2.2. *Key factors in the delivery of CRISPR/Cas9 systems*

2.2.1. *CRISPR/Cas9 loading*

The molecular sizes of plasmids, mRNA, and Cas9 protein are relatively large [22]. Therefore, it is crucial to consider the effective loading of each component when delivering them to the target cell or site. Therefore, it is essential to pay attention to the efficient loading of each component when delivering them to the desired cell or location. Physical delivery methods allow immediate entry of cargo through cell membrane pores, making them less affected by cargo size. However, due to the limited loading capacity of viral vectors, delivering large plasmids exceeding 4 kb presents challenges. Thus, it is necessary to develop delivery methods with high loading capacity. Additionally, since native Cas9 protein carries a positive charge, cationic carriers cannot be used to deliver the native form of Cas9 protein [23]. Therefore, when designing non-viral vectors, the characteristics of the cargo molecules need to be considered and interactions improved through functional modifications of the cargo or the carrier. Furthermore, naked forms of plasmids, mRNA and Cas9 protein are prone to degradation by proteases and nucleases in the bloodstream or recognition and clearance by the mononuclear phagocytic system [24]. Therefore, ensuring the stability of CRISPR/Cas9 in the body is crucial. The design of nanocarriers with polyethylene glycol (PEG) or environment-responsive systems may be potential solutions to address this issue [25,26].

2.2.2. *Targeted CRISPR/Cas9 delivery*

To ensure the effectiveness of the CRISPR/Cas9 system in genetic manipulation and its widespread use in clinical applications, improving its targeting and delivery capabilities is crucial. When using viral vectors to deliver the CRISPR/Cas9 system, there is a risk of random insertion into the host cell genome. Non-viral vectors have gained considerable attention due to their design flexibility and scalability for large-scale production. Currently, ligand-modified chitosan nanoparticles [27] and extracellular vesicles [28] that can bind to specific ligands have significantly enhanced the specificity of the CRISPR/Cas9 system. Furthermore, stimuli-responsive nanoparticles, which can be triggered by intracellular signals such as pH, ATP and glutathione [29–31], enable more precise gene editing, thus achieving targeted delivery of the CRISPR/Cas9 system. Exogenous stimuli-responsive nanoparticles such as light-responsive nanoparticles [32] and magnetic-responsive nanoparticles [33], also show promising applications.

2.2.3. *Transfection of CRISPR/Cas9*

Various transfection methods are commonly used, including liposome transfection, calcium phosphate co-precipitation, microinjection, electroporation and lentiviral vector methods. When utilizing microinjection, electroporation or other methods for gene transfection, it is crucial to take into account the characteristics of the target cells, as well as various parameters and conditions associated with the specific transfection method. These parameters and conditions may include voltage, electrical pulse duration, buffer composition and other relevant factors [34–36]. In lentiviral transfection, several factors contribute to the transfection efficiency, including vector concentration, transduction medium conditions and the characteristics of the target cells and promoters used [37]. Optimizing the transfection strategy is crucial to enhance efficiency while minimizing toxicity to host cells. Additionally, it is essential to establish accurate and effective methods for evaluating transfection efficiency.

3. Common CRISPR/Cas9 delivery strategies

The effective transport of the CRISPR/Cas9 system has garnered considerable interest. Diverse approaches, such as physical methods, viral vector delivery methods and non-viral vector methods, are employed for the delivery of CRISPR/Cas9. Each method possesses distinct advantages and limitations. It is crucial to minimize the risk of off-target effects and develop more efficient and safe delivery methods. Table 2 summarizes several common CRISPR/Cas9 delivery strategies along with their advantages and limitations.

3.1. *Physical delivery*

Currently, physical methods such as microinjection technology, electroporation, and hydrodynamic tail-vein injection (HTVI) are employed for delivering CRISPR/Cas9 systems. Physical delivery methods are relatively straightforward to perform, but are mostly applicable to *in vitro* systems.

3.1.1. *Microinjection*

Microinjection is a highly accurate mechanical methodology that entails the manipulation of a microinjection needle within the visual range of a high-resolution inverted microscope by employing a micromanipulator. It is commonly used for manipulating cells or early-stage embryos. Through microinjection, a variety of substances, including plasmids, mRNA molecules or RNPs, can be directly injected into individual cells. Research conducted by Marcela et al. [37] utilized microinjection to introduce Cas9 mRNA/sgRNA into sheep oocytes, leading to the disruption of the PDX1 gene and investigating its role in pancreatic development. The microinjection technique is easy to control and has minimal off-target effects. The use of a needle allows for the direct injection of cargo with a known molecular weight into the cell, thereby circumventing barriers such as the extracellular matrix, cell membranes and cytoplasm.

Additionally, microinjection technology provides versatility in delivering CRISPR/Cas9 components of various molecular weights and sizes. This characteristic enables the injection of different types of cells and substances, broadening its application in diverse experimental contexts. Researchers have the flexibility to adjust the dosage of materials, facilitating cost control and efficient resource utilization [38]. The microinjection technique can only handle a few hundred cells at a time, resulting in low efficiency and time-consuming procedures [39]. Scott et al. [40] made significant advancements in the traditional microinjection method for

Table 2 – Summary of common CRISPR/Cas9 delivery strategies.

Types of delivery	Delivery strategies	Delivery formats	Advantages	Limitations	Applications	Ref.
Physical delivery methods	Microinjection	DNA; mRNA; Protein	Dosage controllable; Direct delivery	Technical limitation; *In vitro* only; Time-consuming	*In vitro*; *In vivo*	[213]
	Electroporation	DNA; mRNA; Protein	Easy to operate; No cargo size restriction	*In vitro* only; Affects cell viability	*In vitro*;	[214]
	HTVI	DNA; Protein	Easy to operate; Low price	Traumatic to tissue; Low specificity	*In vitro*; *In vivo*	[215]
Viral vector delivery methods	AAV	DNA	Minimal immunogenicity	Limited capacity	*In vivo*	[216]
	AV	DNA	Large capacity; High deliver efficiency	High immunogenicity; Difficult scale production	*In vivo*	[217]
	LV	DNA	Large packaging capacity; Persistent gene transfer	Insertional mutation; Long-lasting expression of Cas9	*In vitro*	[218]
Non-viral vector delivery methods	LNPs	mRNA; Protein	Easy to operate; Low cost	Specific cell tropism	*In vitro*; *In vivo*	[219]
	Polymeric nanoparticles	Protein	Easy to operate	Variable efficiency; Cytotoxicity	*In vitro*	[220]
	Inorganic nanocarriers	Protein	Excellent chemical stability	Variable efficiency depends on cell types	*In vitro*; *In vivo*	[118]
	DNA nanostructure	Protein	Well histocompatibility	Requires modification of template DNA	*In vitro*	[125]

constructing animal models by implementing piezoelectric-driven cytoplasmic microinjection. This approach enabled efficient gene editing in fertilized eggs, effectively addressing the time-consuming nature of microinjection technology in building experimental animal models and improving the survival rate of embryos. In recent years, microinjection has undergone automation through the integration of microinjection techniques with machine technology, leading to further enhancements in the transfection efficiency of mammalian cells. Chen et al. [38] presented a dependable automated microinjection method with high throughput, enabling accurate delivery of CRISPR/Cas9 plasmids into specific cells (Fig. 3A). The emergence of high-throughput automatic microinjection holds promising potential as a more efficient and accurate approach to cell or embryo microinjection [41–43].

3.1.2. *Hydrodynamic tail-vein injection*

The CRISPR/Cas9 system-containing vector is dissolved in physiological saline and then injected into the tail vein rapidly, with the application of high pressure. The rapid flow of physiological saline leads to liver expansion and fluidic transduction of hepatocytes [44]. This technique, known as HTVI, enables convenient and effective gene delivery to mouse hepatocytes and applies to various cell types, making it suitable for *in vivo* injections as well [45]. Niola et al. [46] utilized the HTVI technique to inject CRISPR/Cas9 into the livers of adult mice, demonstrating that the Dnajb1-Prkaca fusion gene is a driving factor in hepatocarcinogenesis. It is noteworthy that the HTVI technique allows for the establishment of a large population of mice with transgenic livers within a remarkably short span of three weeks. This method facilitates the creation of pathophysiological mouse models, providing valuable insights into liver-related diseases. Jeon et al. employed the HTVI method to introduce a CRISPR/Cas9 construct expressing three sgRNA into mice. This led to the development of liver tumors, which were subsequently cultured as organoids resembling tumor formations. These tumor-like organoids were utilized in radiation experiments to assess their sensitivity to radiation [47] (Fig. 3B). Yu et al. [48] proposed an innovative and highly effective strategy for genome editing in the rat liver. They employed the HTVI method to deliver plasmids harboring Cas9 and sgRNA molecules into the rat liver. Their primary objective was to specifically target and modify the Pten gene in hepatocytes, thereby establishing a rat model of non-alcoholic fatty liver disease (NAFLD). Subsequently, this experimental model was utilized to study the fundamental molecular mechanisms and potential pathogenic processes associated with NAFLD. However, it is undeniable that the gene mutation rate of this method is limited, and the injection accumulates in the inferior vena cava, which causes a lot of pressure on the liver of mice, seriously leading to heart rate deceleration and heart failure, so it is not suitable for human clinical use [49]. Indeed, it is important to acknowledge that the transfection efficiency of HTVI technology in tissues other than the liver is generally low. Thus, the utilization of HTVI in other tissues is considerably constrained, with its efficacy mainly demonstrated in the transduction of the CRISPR/Cas9 system into hepatocytes.

3.1.3. *Electroporation*

Electroporation is a widely recognized method for transferring genetic materials into cells. It operates by swiftly disrupting the lipid bilayers comprising the plasma membranes, facilitating efficient intracellular transport. This technique

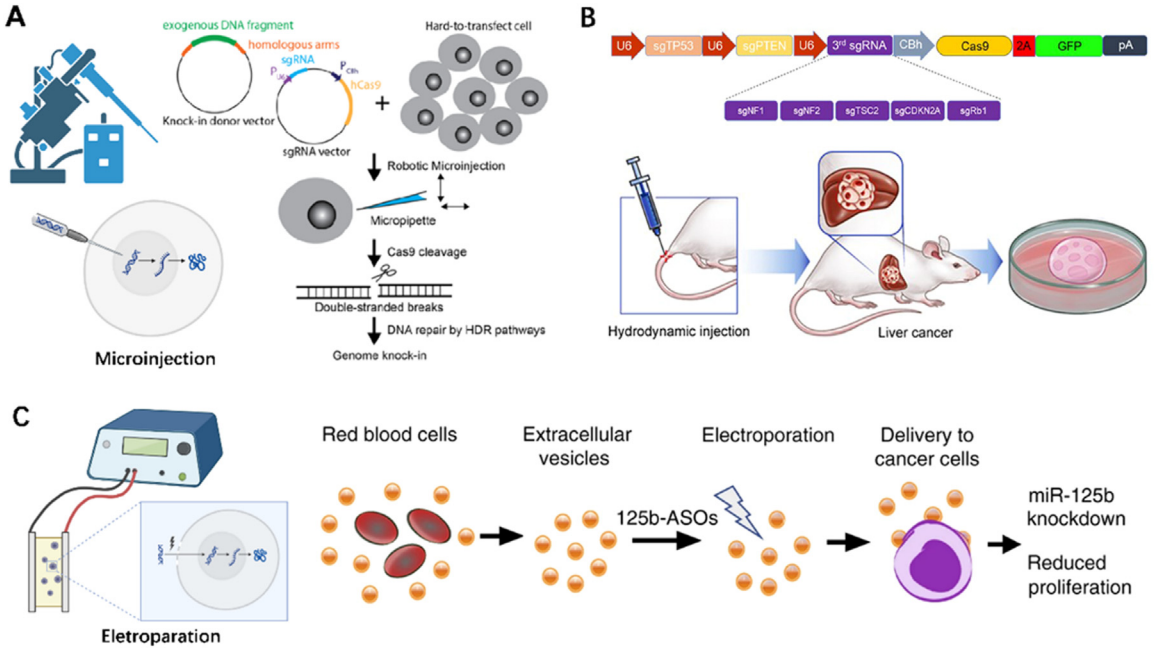

Fig. 3 – Schematic of the physical methods used for CRISPR/Cas9 system delivery. (A) The donor vector containing exogenous DNA fragments and the sgRNA vector containing hCas9 and sgRNA were simultaneously injected into a single hard-to-transfect cell by microinjection technique to achieve HDR of target cells and knock-in of exogenous DNA fragments at specific genomic locations. Adapted with the permission from [38]. Copyright 2022. Institute of Electrical and Electronics Engineers. (B) After establishing a mouse tumor model by intravenous injection of CRISPR-Cas9 constructs via the tail vein, organ tissues were prepared from the generated mouse liver tumors for analysis of their sensitivity to radiation. Adapted with the permission from [47]. Copyright 2023. Technology in Cancer Research & Treatment. (C) RBC-derived EVs loaded with Cas9 mRNA and gRNA were delivered to cancer cells by electroporation for genome editing. Adapted with the permission from [52]. Copyright 2018. Springer Nature.

is particularly advantageous for delivering Cas9, as it supports the delivery of small nanoparticles and molecules measuring a few dozen nanometers [39]. A notable aspect of electroporation is its ability to promptly and effectively destroy the lipid bilayer, resulting in an immediate elevation of membrane permeability. This accelerated membrane damage enhances the transmembrane transport of drugs, establishing a favorable environment for their efficient delivery [50]. Electroporation technology does indeed require specific conditions when used in various cell types, which can lead to higher costs. Researchers often need to optimize the voltage and pulse times of electroporation for specific applications. For instance, Hashimoto et al. [51] established the ideal parameters for incorporating mRNA into mouse zygotes, facilitating extensive genetic analyses in mice. This optimization led to the development of a streamlined, highly efficient and scalable approach for genome editing, eliminating the labor-intensive process of microinjection. As technology continues to advance, the widespread application of electroporation in different environments becomes increasingly significant. Electroporation can deliver a composite system containing CRISPR/Cas9 and vectors *in vivo*. As per the findings presented by Usman et al. [52], red blood cell (RBC)-derived extracellular vesicles (EVs) are suggested as optimal transporters for RNA-based therapeutics. Focusing on the delivery of RNA drugs, the researchers utilized electroporation to encapsulate Cas9 mRNA and gRNA within RBCEV. This innovative approach facilitated the targeted delivery of the aforementioned components to cancer cells, enabling precise genome editing within the desired cellular context. Remarkably, this approach effectively silenced microRNAs in leukemia and breast cancer cells without causing any observable cytotoxicity (Fig. 3C). Laustsen's team utilized Cas9 proteins and chemically modified sgRNA to target cells, demonstrating that chemically synthesized or modified sgRNA could circumvent toxicity issues and significantly enhance the efficiency of gene editing [53]. The use of strong currents can result in cell death, making this technique unsuitable for stress-sensitive cells. Therefore, careful assessment and selection of appropriate conditions are essential when applying electroporation to ensure successful transfection without compromising cell viability.

3.2. *Viral vector delivery*

Viruses play a pivotal role in medical research. In their natural state, wild-type viruses enter the human body and induce diseases due to their pathogenic properties. However, when these viruses are engineered to become non-pathogenic virus particles, their genetic information can still be effectively delivered to host cells through the process of infection [54]. In recent years, lentivirus (LV) vectors, adeno-associated virus (AAV) vectors, and adenovirus (AV) vectors have emerged as popular vehicles for delivering CRISPR/Cas9.

3.2.1. *Lentivirus*

LV vectors possess the capability to accommodate genetic material of approximately 7 kb in size, making them suitable for accommodating the Streptococcus pyogenes Cas9 (SpCas9) gene, which measures around 4.2 kb in size, along with additional sgRNA molecules. The utilization of viral vectors in scientific research does raise specific biosafety considerations, which have stimulated continuous progress in vector technology. Currently, the third-generation LV vector system is commonly referred to as the "self-inactivated" (SIN) LV vector [55]. LV vectors can enable efficient gene transfection, but the issue of altered host tropism has posed a challenge. To overcome this challenge, Lee et al. [56] employed a hepatitis C virus/E1E2-pseudotyped LV vector to carry out targeted delivery of the CRISPR/Cas9 system, specifically targeting the kinesin spindle protein (KSP) gene. This delivery system was implemented *in situ* transplanted tumors within Huh7 mice. This approach successfully resolved the tropism issue associated with LV vectors and achieved the generation of insertion or deletion mutations. When considering sickle cell disease (SCD), gene correction has arisen as a promising gene therapy approach. Uchida et al. [57] devised a non-integrating lentiviral (NILV) vector system, encompassing the delivery of Cas9 protein for both RNA and donor DNA. This innovative system enabled the correction of the SCD mutation occurring in the native βs-globin gene at the protein level. The use of the unintegrated LV vector system facilitated efficient and safe gene delivery and editing, enabling effective insertion mutations without causing long-term genotoxicity in the host (Fig. 4A). However, the preparation of non-genomic integrated LV vectors remains a challenging problem for many laboratories. This obstacle in achieving non-genomic integration is one of the factors contributing to the relatively lower usage of lentiviral vectors in comparison to alternative viral vector systems.

3.2.2. *Adeno-associated virus*

Among the various viral vectors, AAV emerges as the most preferred and extensively utilized vector. The primary rationale behind this preference lies in its non-immunogenic attributes and its capacity to demonstrate serotype specificity upon delivery into the host cell [58]. Additionally, AAV exhibits a neural tropism, ensures persistent gene expression, and is presently regarded as the vector of choice for CRISPR/Cas9 gene editing within the central nervous system [59]. Richards et al. [60] utilized a tropical liver recombinant AAV2/8 vector to deliver a CRISPR/Cas9 recombinant plasmid, successfully correcting the phenylalanine hydroxylase allele (Pahenu2) through homologous recombination to treat phenylketonuria. This approach addressed the limitations of dietary therapy by utilizing gene therapy. Similarly, in the same year, Croci et al. demonstrated the precise repair of Forkhead Box G1 (FOXG1) gene variants associated with Rett syndrome using the AAV-coupled CRISPR/Cas9 system [61]. This significant breakthrough further propelled the advancement of gene therapy in the context of Rett syndrome.

The AAV system provides durable gene expression, leading to lasting therapeutic effects. However, the extended-expression of AAV vectors may potentially give rise to safety issues, such as cellular toxicity, off-target effects, and immune reactions. To overcome these difficulties, Li and colleagues [62] introduced an inventive integrated self-deleting AAV-CRISPR-Cas9 system. This system incorporates the Staphylococcus aureus Cas9 nuclease (SaCas9) and sgRNA. The Cas9 protein in this system not only facilitates the cleavage of the intended PCSK9 gene but also cleaves the AAV vector itself. This ingenious approach prevents excessive self-expression, substantially mitigates off-target effects, and thereby enhances safety (Fig. 4B). AAV is already extensively used as a delivery vehicle in various fields, and the transition from *in vitro* trials to clinical trials is forthcoming [63]. Multiple studies have revealed that the immune response against AAV and Cas9 is a major barrier to AAV-mediated delivery of the CRISPR/Cas9 system for human gene editing. The presence of immune responses can result in unfavorable outcomes, including the cessation of the delivery procedure, disturbance of modified cells, and the induction of inflammation within the organism [64,65]. In a groundbreaking study, Hakim's team investigated the immunological consequences of AAV-mediated Cas9 expression in large mammals. They administered intramuscular and intravenous injections of AAV-CRISPR to Duchenne muscular dystrophy (DMD) dogs. While the experimental dogs showed recovery of the muscular dystrophy protein after treatment, it also resulted in muscle inflammation and immune responses. These findings highlight that the immune response of large mammals to Cas9 is a major obstacle for AAV-CRISPR therapy [66]. To address the immune response issue caused by AAV and Cas9 proteins, different AAV serotypes or Cas9 homologous genes can be utilized [70]. Additionally, establishing a sensitive monitoring platform for the AAV-CRISPR system can provide real-time insights into the host immune response triggered by exogenous components of AAV-CRISPR [67]. By doing so, scientists would be able to enhance their comprehension of and address potential immune responses, thus enhancing the safety and efficiency of the AAV-CRISPR system when used for human gene editing.

3.2.3. *Adenovirus*

Currently, AVs are widely used in clinical trials for gene editing [68]. AVs have linear double-stranded DNA genomes, with a loading capacity of 8 kb. As non-enveloped viruses, they lack an outer lipid membrane, which reduces the risk of integration into the host genome and decreases the occurrence of off-target effects in genome editing. An example illustrating this is the treatment of Hemophilia B, an inherited disease caused by a mutation in the Factor IX (FIX) gene. Stephens et al. [69] utilized AVs as vectors to deliver the CRISPR/Cas9 system, targeting the FIX gene and enabling the insertion of the mFIX gene in the ROSA26 safe harbor, resulting in long-term correction of Hemophilia B (Fig. 4C). AVs have undergone significant advancements over time. The current generation of AV vectors, known as third-generation vectors, consists of high-capacity adenovirus (HCAdV) or helper-dependent adenovirus (HDAdV). However, it is important to note that the presence of the CRISPR/Cas9 system in target cells can lead to off-target effects, cytotoxicity, or activation of the host immune response. To address these safety concerns, Palmer's team [70] developed a

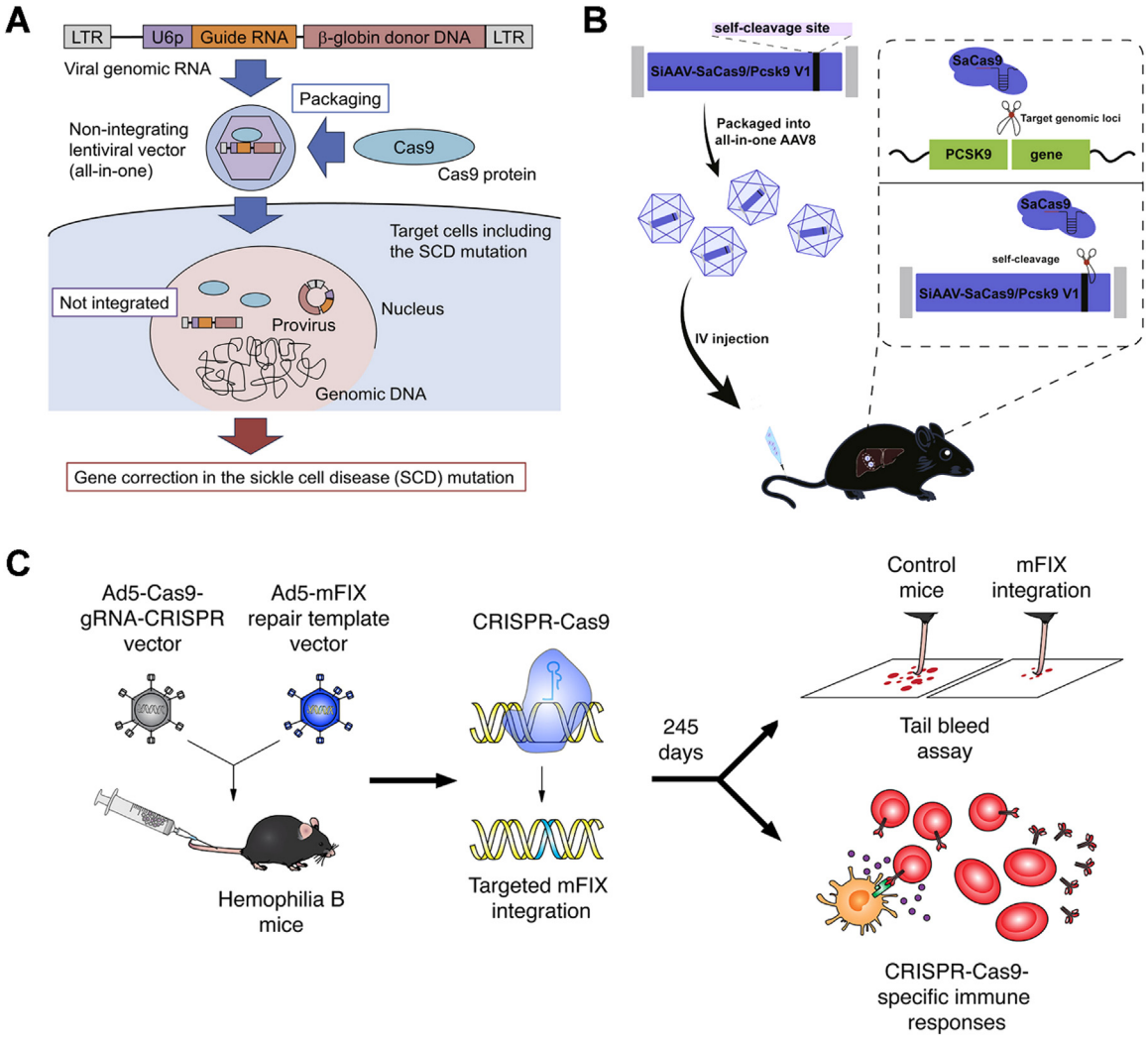

Fig. 4 – Schematic diagram of viral vector delivery of CRISPR/Cas9 system. (A) Schematic diagram of the assembly process of a non-integrating lentiviral vector and genetic correction of SCD gene mutations. Adapted with the permission from [211]. Copyright 2021. Elsevier. (B) Schematic diagram of the assembly process of the integrated self-cleavage AAV-CRISPR-Cas9 system and the *in vivo* gene editing and vector self-cleaving process. Adapted with the permission from [62]. Copyright 2021. Elsevier. (C) A single injection of Cas9/sgRNA loaded with an AAV vector achieved the knock-in of the mFIX gene in the ROSA26 safe harbor locus. The integrated mFIX gene on the targets and the acquired immune response induced by the AV vector and Cas9 nuclease were detected 245 d after injection. Adapted with the permission from [69]. Copyright 2019. Elsevier.

CRISPR/Cas9-mediated self-cleavage HDAdV by incorporating the target sequence encoded by the helper virus and production cell into the Cas9 encoded by the HDAdV. This innovative approach effectively reduced the inhibitory activity of Cas9 proteins and mRNA, addressing the aforementioned safety concerns and enhancing the safety of AVs-mediated CRISPR/Cas9 technology. HDAdV stands out as the predominant gene delivery vector employed in the treatment of lung diseases. This preference arises from HDAdV's ability to package all the necessary components for achieving efficient site-specific gene integration [71,72]. Unlike other vectors, HDAdV possesses minimal original viral DNA sequence and boasts an impressive encapsulation capacity of up to 35 kb. This exceptional capacity allows a single HCAdV viral vector to deliver CRISPR/Cas9 and concurrently encapsulate multiple genes, such as fluorescent or luminescent reporters [73]. In comparison to the approach of using multiple vectors to deliver genetic components, utilizing a single vector directly can significantly enhance transduction efficiency and reduce the occurrence of host immune responses. The formation of covalently closed circular DNA (cccDNA) during hepatitis B virus (HBV) replication contributes to persistent HBV infection, making it a global health concern. The expansive loading capacity of HCAdV offers a promising solution for HBV genome editing. Schiwon et al. [74] incorporated the entire nuclease system along with a chimeric gRNA sequence of the CRISPR/Cas9 system into the HCAdV Cas9 expression cassette. This strategy involved cloning three gRNAs that were expressed in HCAdV and the Cas9 expression cassette into a shuttle

vector, which was then transferred to the HCAdV production vector. Their research successfully targeted and degraded the HBV genome, including HBV cccDNA. Furthermore, apart from using third-generation AV vectors, the utilization of the CRISPR/Cas9 system to modify the AV genome or optimize AV vector delivery and gene editing techniques can enhance transfection efficiency and streamline the gene editing process in human T cells.

Without a doubt, the present viral vectors commonly used for delivering CRISPR/Cas9 components possess certain drawbacks, including mutagenicity, immunogenicity and lack of precision in targeting. Although viral vectors exhibit high efficiency in delivering CRISPR/Cas9, overediting can raise safety concerns. Conversely, non-viral vectors have significant potential in overcoming the limitations associated with viral vectors [39].

3.3. *Non-viral vector delivery*

Non-viral vectors are increasingly gaining favor as the preferred method for delivering CRISPR/Cas9, primarily because they can effectively overcome the drawbacks of oncogenicity and immunogenicity typically associated with viral vectors. Non-viral vectors offer multiple benefits, including a high capacity to carry genetic material, improved safety, convenient accessibility, and the potential for large-scale production. Currently, numerous reviews have provided comprehensive summaries of their applications in cancer treatment and genetic disease therapy [75,76]. These reviews highlight that with advancements in nanotechnology, the development of more controllable, precise and safe nanocarriers holds great clinical translational potential. Non-viral vectors are anticipated to have a greater impact in the fields of cancer treatment, genetic disease therapy, and the study of molecular mechanisms.

3.3.1. *Polymer nanoparticles*

Polymeric vectors, such as synthetic vectors, exhibit immense promise in the realm of CRISPR/Cas9-based gene editing [27,69]. Positively charged polymers can encapsulate CRISPR/Cas9 through electrostatic interactions with negatively charged nucleic acids, facilitating their cellular uptake via reticular protein-mediated endocytosis. Cationic polymers and cationic lipids represent examples of positively charged carriers that can be employed for gene delivery [77].

Ban et al. [78] devised a supramolecular substrate-mediated delivery (SNMD) approach by leveraging existing supramolecular nanoparticle (SMNP) carriers. They encapsulated RNP onto SMNP carriers, leading to the formation of RNP⊂SMNPs through self-assembly. In this platform, the silicon nanowire substrate (Ad-SiNWS), modified with adamantane (Ad), served as a means of assembling and locally concentrating the SMNPs. The physical interaction between cells and SMNPs facilitated the uptake of RNP⊂SMNPs, enabling efficient gene editing mediated by CRISPR/Cas9. The study employed the U87 glioblastoma (GBN) cell line as a model system, and the uptake parameters of SMNP containing RNP were further evaluated based on green fluorescent protein (GFP) expression (Fig. 5A). In a separate work, by Deng et al. [79] developed a delivery system for Cas9/sgRNA, wherein the release of Cas9/sgRNA was triggered by near-infrared and reduction responses of nanoparticles. They encapsulated the antitumor photosensitizer chlorin e6 (Ce6) within micelles modified with nitrilotriacetic acid, forming a responsive stimulation system with His-labeled Cas9 RNP. Upon near-infrared stimulation, Ce6 generated reactive oxygen species (ROS) that were released from lysosomes into the cytoplasm. Subsequently, disulfide bonds were reduced, facilitating the release of Cas9/sgRNA. Cas9/sgRNA was designed to target the antioxidant gene Nrf2 and synergistically enhanced the sensitivity of tumor cells to ROS for photodynamic therapy (Fig. 5B). Chou et al. [80] performed CRISPR/Cas9-mediated Retinoschisin 1 (RS1) knockout in patients with X-linked juvenile retinal splitting (XLRS) by intravitreal or subretinal injection of SMNP vectors loaded with CRISPR-Cas9 plasmids and the therapeutic RS1, providing a revolutionary treatment option for patients with XLRS (Fig. 5C). However, the delivery efficiency was low and the gene insertion-deletion was serious. Based on these studies, Wang et al. [81] performed genome editing using PEG nanoparticles (P-HNPS) and Cas9-expressing plasma sgRNA, which greatly improved the Cas9 transfection efficiency and uptake of sgRNA. Compared with existing polycation-based gene delivery, P-HNPS have great potential for non-viral delivery. Li et al. [82] synthesized semiconductor polymer brushes (SPPF) through the sequential conjugation of alkyl side chains, PEG chains and fluorinated polyethylenimine (PF) onto the backbone of initial semiconducting polymers (SPs). Subsequently, they encapsulated dexamethasone (Dex) within the SPPF structure. Finally, SPPF was combined with plasmids to form SPPF-Dex/PDNA nanoparticles. The alkyl side chains formed a hydrophobic core, the PEG chains provided biocompatibility to the nanoparticles, and PF facilitated binding to the CRISPR/Cas9 complex. Dex facilitated the enlargement of nuclear pores, thereby promoting the entry of plasmids into the nucleus. The release of SPPF-Dex/PDNA nanoparticles was observable via near-infrared imaging, and it was demonstrated that efficient gene editing could be achieved using this approach. This development shows great potential as a novel approach for precise gene editing through the use of CRISPR/Cas9 (Fig. 5D). Dendritic polymers are artificially synthesized hyperbranched nanostructures with numerous branches radiating outward from a central core. Their size and structure can be modified, making them suitable for drug delivery [83]. Protein surfaces typically display cationic groups such as amines, imidazoles and guanidines, as well as anionic carboxylate groups. Experimental evidence has demonstrated that dendritic polymers containing phenylboronic acid can form complexes through nitrogen-boronate coordination, cation-π interactions and ionic interactions. These interactions result in a high affinity for RNPs and facilitate the efficient delivery of the Cas9 protein to various cell lines. Consequently, CRISPR/Cas9 editing efficiency within cells is significantly improved [84]. Among commercially available non-viral vectors based on dendritic polymers, poly(amidomine) dendrimers (PAMAM) dendrimers are the most widely used. Kretzmann et al. [85] employed a highly controlled synthesis strategy using click chemistry

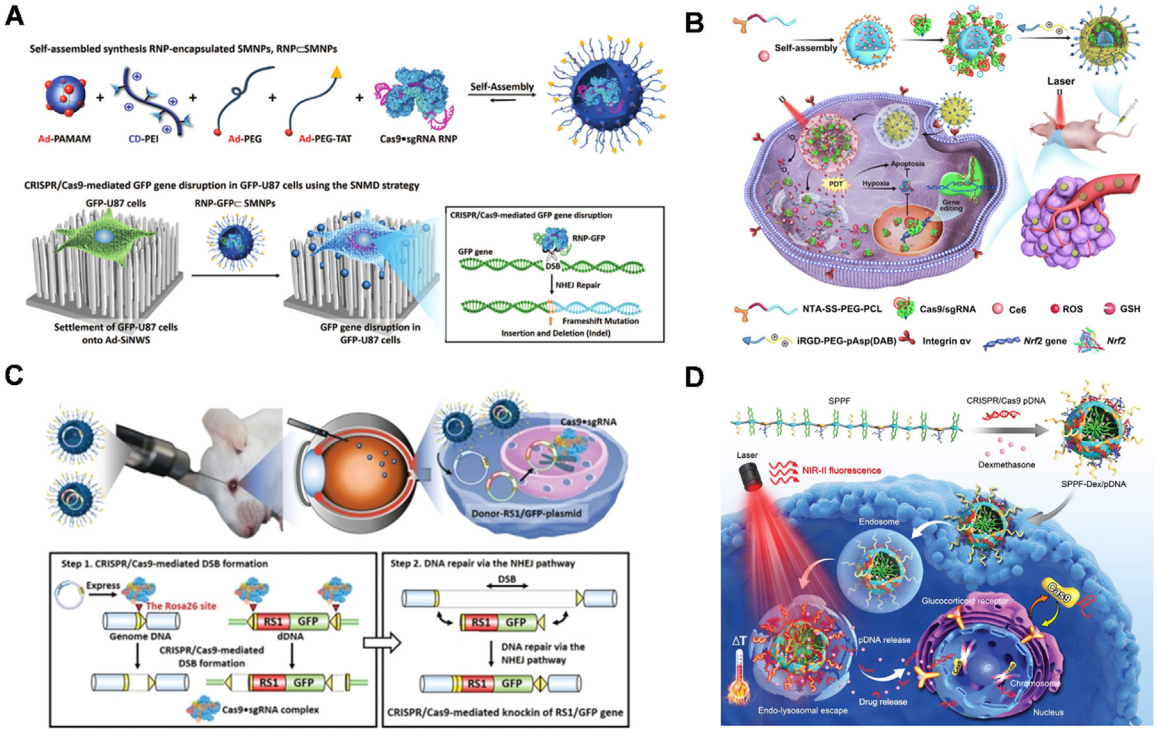

Fig. 5 – Schematic diagram of polymers delivery of CRISPR/Cas9 system. (A) Schematic diagram of the delivery of Cas9 protein and sgRNA targeting GFP into the GFP-U87 cell lines for disruption of the GFP gene by the self-assembled formation of RNP⊂SMNPs. Adapted with the permission from [78]. Copyright 2021. John Wiley and Sons. (B) Schematic diagram of the preparation, delivery, and intracellular delivery of nanoparticles containing Nrf2-targeted Cas9/sgRNA and the antitumor photosensitizer Ce6. Adapted with the permission from [79]. Copyright 2020. American Association for the Advancement of Science. (C) SMNP vectors were developed for co-delivery of Cas9/sgRNA plasmids and donor RS1/GFP plasmids to mediate DSB and repair via the NHEJ repair pathway in the mouse retina for RS1 knockin. Adapted with the permission from [80]. Copyright 2020. John Wiley and Sons. (D) Schematic diagram of the preparation of semiconductor polymer brushes-dexamethasone (SPPF-Dex) nanoparticles for CRISPR/Cas9 delivery and intracellular genome editing process under 808 nm laser irradiation. Adapted with the permission from [82]. Copyright 2019. John Wiley and Sons.

to design dendritic copolymers, which exhibited promising outcomes in terms of transfection efficiency and packaging capacity. Zhang et al. [86] have developed a zwitterionic polymer with a branched structure (ZEBRA) for CRISPR/Cas9 delivery. This ternary copolymer is composed of agarose (AG), low molecular weight polyethylenimine (PEI), and hyaluronic acid (HA). It exhibits high transfection efficiency, specific targeting capabilities, and excellent biocompatibility.

3.3.2. *Lipid nanoparticles*

Nanoparticles serve as effective carriers for delivering encapsulated drugs into organisms through cell membranes via endocytosis [87]. Among various nanoparticles, lipid nanoparticles (LNPs) are extensively utilized as nanocarriers for delivering drugs and nucleic acids, owing to their straightforward preparation, convenient surface modification, and favorable biocompatibility [88]. In numerous investigations centered around the transportation of CRISPR/Cas9 utilizing LNPs, scientists are persistently working towards the development of delivery systems that are both more effective and safer. In terms of safety, Suzuki et al. [89] successfully constructed an LNP-mediated RNP delivery platform by optimizing the synthesis conditions. This platform enabled gene destruction and base substitution, thereby avoiding issues related to DNA cleavage and high concentrations of Cas enzymes, with minimal cytotoxicity. Additionally, Wei et al. [90] demonstrated that LNPs could effectively transport RNPs to specific cells and tissues through rational design. Overall, ionizable cationic liposomes, zwitterionic phospholipids, cholesterol, and PEG liposomes are essential components of traditional liposome formulations. Building upon this foundation, the inclusion of permanent cationic lipids helps maintain the fundamental tertiary structure and stability of RNPs. Moreover, by utilizing low-dose intravenous injection, organ-specific cancer models can be efficiently established in the lung and liver of mice (Fig. 6A). Liu et al. [91]. developed a novel delivery method for CRISPR/Cas9 by encapsulating Cas9 mRNA and sgRNA into disulfide-integrated bio-reducible LNPs (BAMEA-O16B). These LNPs exhibited a high release efficiency of Cas9 mRNA and sgRNA when exposed to the intracellular environment, thereby enabling successful gene editing. *In vitro* experiments demonstrated the ability of these nanoparticles to achieve up

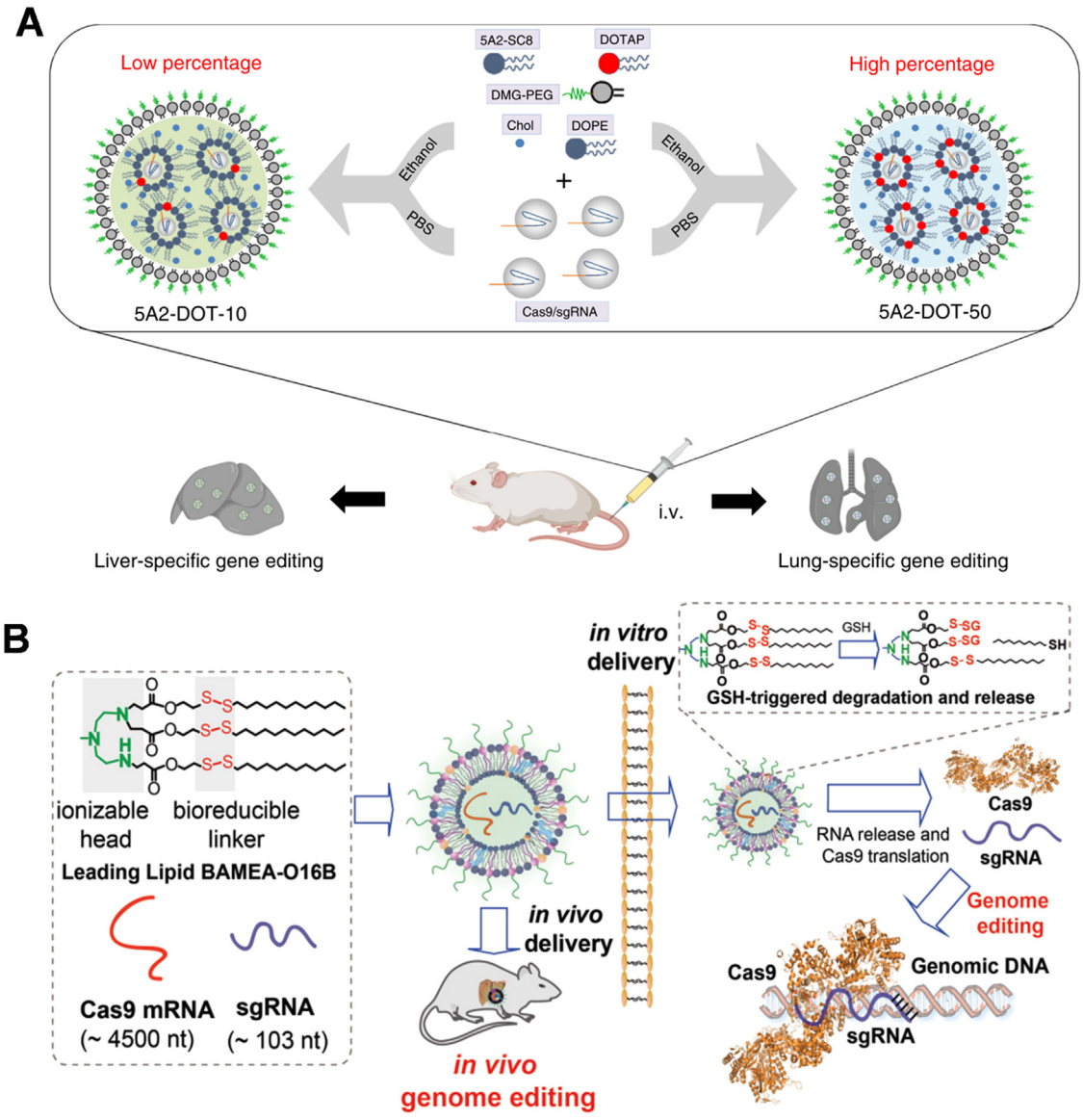

Fig. 6 – Schematic diagram of LNP delivery of CRISPR/Cas9 system. (A) Permanent cationic supplements were added to the traditional LNP formulations to maintain the basic tertiary structure and stability of RNPs, and low-dose intravenous injection of mice could effectively establish lung and liver organ-specific tumor models. Adapted with the permission from [90]. Copyright 2020. Nature Portfolio. (B) Schematic diagram of the preparation of bio-reducible lipid/Cas9 mRNA/sgRNA nanoparticles for delivery of CRISPR/Cas9 system *in vitro* and *in vivo*. Adapted with the permission from [91]. Copyright 2019. John Wiley and Sons.

to 90% reduction in GFP expression in cells and 80% reduction in PCSK9 expression. BAMEA-O16B has emerged as a versatile and effective nanoparticle platform for CRISPR/Cas9 genome editing, establishing itself as one of the most promising systems for delivering CRISPR/Cas9 (Fig. 6B). Another notable advancement was made by Hajj et al. [92] through the development of branched-tail LNPs. These LNPs can deliver multiple mRNAs, offering great flexibility for CRISPR/Cas9-mediated polygene editing. Moreover, it was demonstrated for the first time that ionizable LNPs can encapsulate and deliver mRNA with diverse functions within the same formulation. This breakthrough opens up possibilities for treating diseases caused by multiple defective proteins. Additionally, lactate dehydrogenase A (LDHA) is a crucial target in metabolic engineering. Ju et al. [93] designed cationic lipid nanoparticles for co-delivering Cas9 and sgRNA plasmid DNA to enable gene editing of LDHA. When combined with PD-L1 antibodies, editing the LDHA gene activates T cells and results in a synergistic anti-tumor effect, which provides a combined strategy for tumor therapy by integrating tumor microenvironment metabolic engineering with immune checkpoint inhibition.

Exosomes are small lipid bilayer vesicles released by cells in various physiological and pathological conditions [94]. They are present in a stable form in plasma and urine. These vesicles play a crucial role in intercellular

communication and can transport exogenous substances like proteins, nucleic acids and lipids to specific cells or tissues for targeted drug delivery [95]. Compared to liposomes, metal nanoparticles, polymers and other synthetic drug delivery systems, the naturally occurring nature of exosomes provides a significant advantage [96]. Exosomes have a better ability to penetrate tissues and the bloodstream, and they can even traverse biological barriers like the blood-brain barrier [97]. It has been observed that exosomes derived from tumor cells exhibit minimal immunogenicity and toxicity, making them suitable natural carriers for delivering CRISPR/Cas9 to tumor cells for gene editing purposes [98,99]. Additionally, exosomes contain various transmembrane and membrane-anchored proteins that enable efficient delivery of cargo to recipient cells while evading degradation by phagocytic clearance [100]. These remarkable properties make exosomes a promising platform for CRISPR/Cas9 delivery. By modifying the surface of exosomes, their targeting ability can be enhanced. For instance, Ye et al. [101] engineered a functionalized exosome (M-CRISPR-Cas9 exosome) by combining GFP and nanoparticles with extracellular membrane proteins CD63 and Cas9, respectively. The affinity between GFP-GFP nanoparticles allowed selective capture and loading of Cas9 proteins into exosomes, which were then combined with sgRNA to form ribonucleoprotein complexes for delivery to target cells. However, exosome-based delivery has limitations in transporting large nucleic acids or high molecular weight drugs, such as miRNAs and siRNAs, and exosomes face challenges related to low cargo loading during delivery. To address these limitations, Lin et al. [102] pioneered the use of a hybrid exosome-liposome system for delivering the CRISPR/Cas9 system to mesenchymal stem cells. The delivery of CRISPR/Cas9 into cells and subsequent release for gene editing purposes resolves the challenge of loading large nucleic acids into exosomes. However, it has been acknowledged that the release of sgRNA and Cas9 proteins mediated by exosomes can potentially impact the surrounding and distant tissues or cells through cellular communication pathways. This further complicates the issue of off-target effects and safety associated with the CRISPR/Cas9 system [94]. Consequently, additional research and development are necessary to ensure the safe and effective clinical application of the CRISPR/Cas9 system mediated by exosome vectors.

3.3.3. *Inorganic nanocarriers*

Inorganic nanomaterials possess a large specific surface area, customizable surface functionalization and adaptable size, rendering them exceptionally flexible in this domain. Among them, gold nanomaterials have garnered significant interest due to their unique nanostructures, exceptional photothermal effects, and diverse surface chemical properties [103,104].

Gold nanoparticles (AuNPs) are commonly encapsulated by polymers and conjugated with various ligands, providing them with additional biological properties such as enhanced stability, biocompatibility, and transfection capabilities [105]. Chen et al. [106] developed gold nanorod (AuNR)-PEI (ARP) vectors by utilizing a layer-by-layer assembly approach to bind branched PEI with AuNPs for mediating CRISPR/Cas9 transfection in host cells. Cells efficiently internalized the AuNP-PEI vectors via a caveolae-dependent pathway and effectively evaded endosomal entrapment, thanks to the proton-sponge effect. Similarly, Wang et al. [107] presented a multifunctional vector comprising a lipid/AuNPs complex for delivering Cas9/sgRNA to the PLK-1 gene. The AuNPs served as carriers and photothermal release agents. TAT peptides were incorporated into the vector to facilitate plasmid binding and enable nuclear targeting. Moreover, the external lipid membrane of the vector preserved the integrity of the Cas9-sgPlk-1 plasmid and facilitated proficient cellular uptake. The system was released intracellularly in the presence of light and heat to achieve knockdown of the PLK-1 gene (Fig. 7A).

Gold nanoclusters (AuNCs) are ultra-small nanoparticles comprised of several to hundreds of gold atoms, with metal nuclei smaller than 2 nm. In comparison to traditional AuNPs, AuNCs offer several advantages such as improved synthesis, stability, biocompatibility, and efficient renal clearance [108]. Photothermal therapy (PTT) aims to treat tumors by raising their temperature above 50 °C, but this approach often leads to hyperthermia and damage to healthy tissues. To minimize thermal damage to adjacent normal tissues, a gentle PTT strategy has been devised to overcome the heat resistance of tumors. Research has shown that by inhibiting heat shock protein 90 alpha (HSP90α), which functions as a chaperone protein for heat shock proteins (HSPs), the heat resistance of cancer cells can be reduced, offering the potential for achieving tumor-specific low-temperature hyperthermia. Li et al. [109] introduced a hypoxia-activated CRISPR-Cas9 nanosystem (APACPs) using AuNRs as a foundation. They employed a hypoxia-responsive azobenzene linker to covalently attach the CRISPR system to the AuNRs. Under near-infrared light exposure, the AuNRs generated intracellular heat. The hypoxic condition triggered the cleavage of azo bonds, enabling the controlled release of CRISPR-Cas9 for subsequent gene editing. By specifically targeting the knockout of the HSP90α gene, the researchers diminished the heat resistance of cancer cells. Their approach achieved targeted mild hyperthermia and synergistic anticancer effects in both *in vitro* and *in vivo* experiments (Fig. 7B). Ju et al. [110] made an initial breakthrough by demonstrating that the self-assembled complex of AuNC and SpCas9 (SpCas9-AuNC) exhibited efficient transport of the SpCas9 protein to the nucleus. The SpCas9-AuNC complex displayed stability at higher pH levels but underwent decomposition at lower pH levels (Fig. 7C). Leveraging the decomposition process of SpCas9-AuNC, SpCas9 could be successfully delivered to the cells and nucleus, enabling its function in gene editing.

Superparamagnetic iron oxide nanoparticles (SPIONs) have emerged as a groundbreaking agent for the diagnosis and treatment of cancer [111]. Magnetic drug targeting utilizing SPIONs enables the precise delivery of therapy under the control of an external magnetic field, offering the advantages of high effectiveness, efficiency, and minimal side effects [112]. PEI-coated SPIONs have exhibited the potential to activate endogenous promoters effectively while delivering CRISPR/Cas9 into human foreskin fibroblast cells, with reduced cellular toxicity attributed to the PEI coating [113]. An additional study has also highlighted the potential

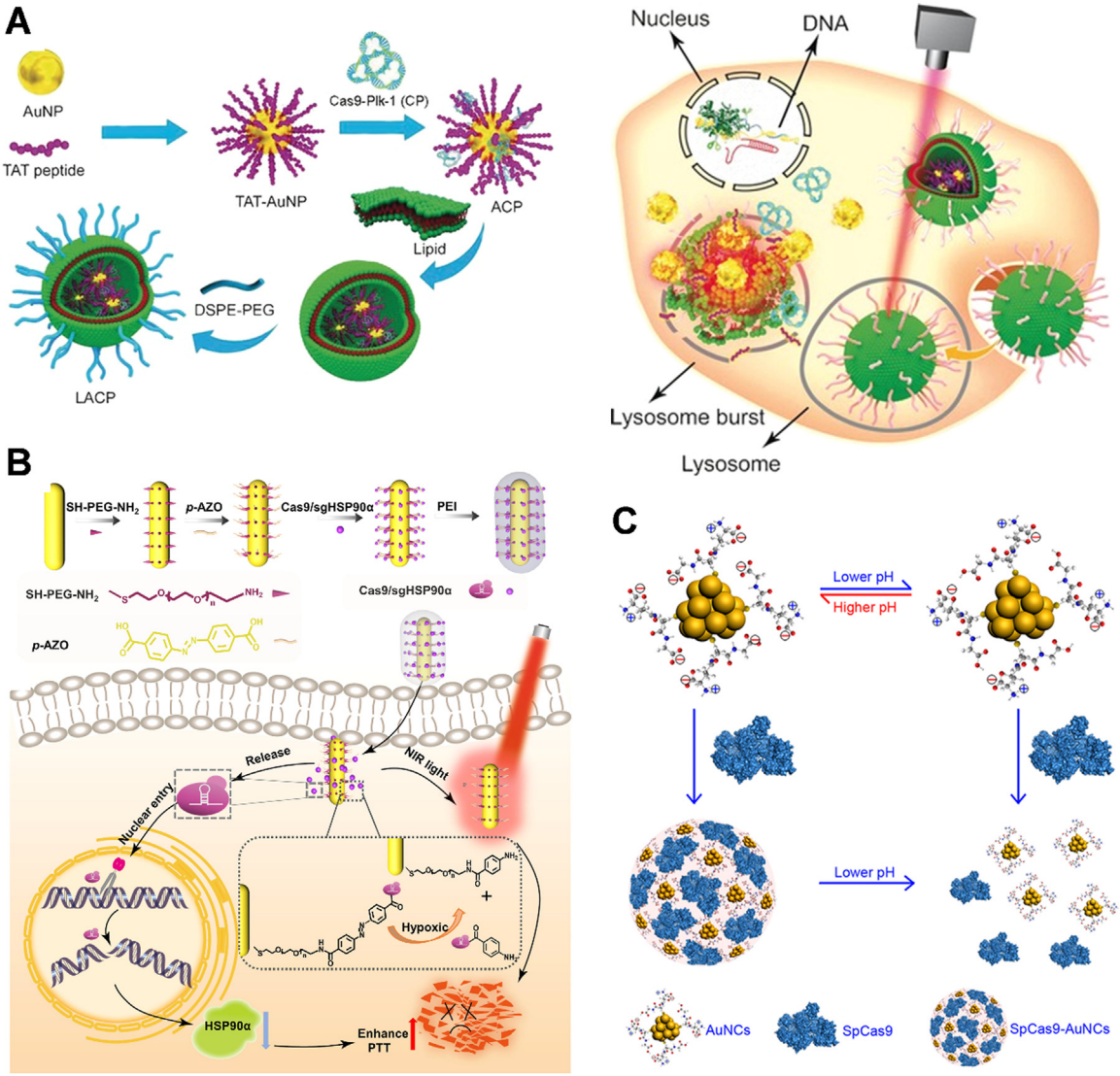

Fig. 7 – Schematic diagram of gold nanomaterials delivery of CRISPR/Cas9 system. (A) Schematic diagram of the preparation of a multi-functional delivery system based on lipid/AuNPs complexes and knockdown of A375 cell target genes under laser enhancement. Adapted with the permission from [107]. Copyright 2018. John Wiley and Sons. (B) Schematic diagram of the preparation of the APACPs based on AuNRs and delivery of Cas9-sg HSP 90α to the nucleus for gene editing and photothermal therapy under hypoxia triggering. Adapted with the permission from [109]. Copyright 2021. John Wiley and Sons. (C) Schematic diagram of pH-induced assembly and decompression of SpCas9-AuNC. Adapted with the permission from [110]. Copyright 2019. American Chemical Society.

of PEI-modified magnetic nanoparticles for the delivery of CRISPR/Cas9 plasmids into cells without the need for transfection reagents such as lipofectamine. This approach successfully mediates NHEJ or HDR of DNA after cellular internalization [114].

Mesoporous silica nanoparticles (MSNs) possess a remarkable capacity for loading various substances, and their expansive surface area enables straightforward customization, rendering them ideal for the development of drug delivery systems featuring targeting capabilities and triggered drug release. They can evade endocytic pathways and selectively release drugs in cells or tissues [115]. García-Fernández et al. [116] developed a multifunctional platform that simultaneously loaded an anti-inflammatory drug, VX-765 and CRISPR/Cas9 plasmids. They edited the gene gasdermin D, which is associated with inflammation-induced cell death, thereby enhancing the anti-inflammatory effect. MSNs show great promise for the simultaneous delivery of CRISPR-Cas9 machinery and drugs. MSNs modified with nucleus-targeting sequences (NLS) facilitate the transport of CRISPR/Cas9 plasmids to the nucleus, offering effective protection against degradation. This intracellular delivery system presents an appealing approach to overcome the physiological barriers associated with CRISPR/Cas9 delivery [117]. Traditional MSN particles face challenges in protein delivery, such as low loading efficiency and susceptibility

to degradation. Liu et al. [118] improved the gene editing efficiency by enhancing the interaction between Cas9 protein and MSN carriers through modification of protein groups.

3.3.4. *DNA nanocarriers*

Currently, a diverse range of DNA nanostructures, including DNA nanoflowers (DNFs), DNA nanospheres, DNA nanotubes, and others, are being designed for various biomedical applications. Additionally, DNA probes, DNA nanochannels, and DNA templates can undergo structural transformations when exposed to specific stimuli, presenting potential opportunities in drug delivery and molecular assembly [119]. DNA nanostructures have gained significant attention in biomedical fields such as targeted drug delivery [120] and biological imaging, owing to their impressive characteristics such as high drug-carrying capacity, biodegradability, ease of functionalization, and excellent biocompatibility. Ding et al. [121] were the pioneers in developing a CRISPR/Cas9 delivery platform using non-cationic DNA crosslinked nanogels. In their platform, DNA was modified with polycaprolactone brushes (DNA-g-PCL) and loaded with a Cas9/sgRNA complex. Subsequently, the DNA conjugate was crosslinked through nucleic acid hybridization to form a nano-hydrogel, effectively encapsulating and protecting the CRISPR-Cas9 system. This delivery platform not only safeguards the CRISPR-Cas9 system from degradation but also demonstrates efficient editing of target genes. Additionally, this platform holds great potential for cytoplasmic protein delivery and enhanced gene editing, as it is designed to precisely deliver its cargo to the intended target site. miRNAs, which play a significant role in regulating the genome in both physiological and pathological conditions, served as the inspiration behind Shi et al.'s [122] development of a miRNA-regulated CRISPR/Cas9 delivery platform. To achieve this, they synthesized DNFs using RCR techniques, incorporating both the MUC1 aptamer and miR-21 binding sequences into the DNF structure [123]. The utilization of DNFs in the miR-21-responsive Cas9/sgRNA RNP delivery system enables efficient delivery of the Cas9/sgRNA complex. The complex is loaded onto the DNFs through sequence hybridization. This system incorporates MUC1 aptamers, which facilitate the escape of the loaded cargo from lysosomes. Furthermore, miR-21 serves as a genome regulation tool in this system. In the designed delivery system, the Cas9/sgRNA complex is initially contained within the DNFs. Nonetheless, through the utilization of a foothold-mediated sequence, the Cas9/sgRNA complex can be liberated from the DNFs and effectively transported to the nucleus (Fig. 8A). Compared to a non-miR-21 responsive control group, this miR-21-responsive delivery system demonstrates higher gene editing efficiency, indicating its effectiveness in precisely targeting and editing specific genes. Additionally, Li et al. [124] introduced a DNA-based nanosystem activated by protons to facilitate the simultaneous delivery of Cas9/sgRNA and DNAzyme. Within this system, a lengthy single-stranded DNA serves as the structural foundation of the nanosystem, encompassing the sgRNA recognition sequence of CRISPR/Cas9, the DNAzyme responsible for mRNA cleavage, and an HhaI cleavage site. Through the assistance of the DNAzyme cofactor Mn^{2+}, the DNA chain is compacted into nanoparticles. Within the lysosome, the presence of protons triggers the decomposition of the polymer coating encompassing the nanoparticles, revealing the HhaI enzyme. This enzyme identifies and cleaves the specific cleavage site, thereby liberating Cas9/sgRNA and DNAzyme. Consequently, this efficient regulation of gene expression yields a notable therapeutic effect, particularly evident in the context of breast cancer. Sun et al. [125] addressed the challenges associated with the conventional Watson-Crick base-pairing method for DNA nanostructure assembly by optimizing the synthesis process and introducing a novel approach using rolling RCA. This method allowed for the efficient synthesis of a DNA nanostructure known as a DNA nanoclew. In their study, the researchers designed palindromic sequences and incorporated partial sgRNA compensation, enabling the loading of Cas9 RNP onto the DNA nanoclew through base complementary pairing. To enhance the escape from endosomes, the DNA nanoclew was additionally coated with PEI. To efficiently deliver the Cas9 RNP to the nucleus for gene editing purposes, the scientists integrated N-terminal and C-terminal nuclear localization signal peptides with Cas9. Through this fusion construct, successful transportation of Cas9 RNP to the nucleus was achieved, leading to efficient knockout of the EGFP gene. The study also included a control experiment to compare the delivery efficiency of Cas9 RNP using the nanoclew carrier against delivery with only PEI. The findings revealed that the delivery efficiency of Cas9 RNP with the nanoclew carrier was approximately seven times higher than that of the control group utilizing only PEI. Overall, the research conducted by Sun et al. introduced a highly effective carrier for delivering Cas9 RNP. The incorporation of optimized methods for synthesizing DNA nanostructures using rolling circle amplification, the design of palindromes, partial compensation of sgRNA, and the addition of a PEI coating to facilitate endosomal escape jointly contributed to the heightened delivery efficiency and successful gene editing achieved through the Cas9 RNP-loaded nanoclew carrier (Fig. 8B).

4. Biomedical applications of CRISPR/Cas9 system

The CRISPR/Cas9 technology holds vast potential in the realm of biomedicine. This powerful technology enables precise modifications of the genome, offering essential tools for studying disease mechanisms and developing therapeutic approaches. Establishing disease models enables detailed investigations into disease progression and the testing of potential treatment methods. Moreover, CRISPR/Cas9 is invaluable for large-scale gene functional studies and genome-wide screenings. The versatility of the CRISPR/Cas9 system extends beyond basic research, as it holds tremendous potential in various areas, including gene therapy, infectious disease diagnostics and bioengineering (Fig. 9).

4.1. *Disease treatment*

Cancer represents a major menace to human well-being and stands as the second highest contributor to mortality [126].

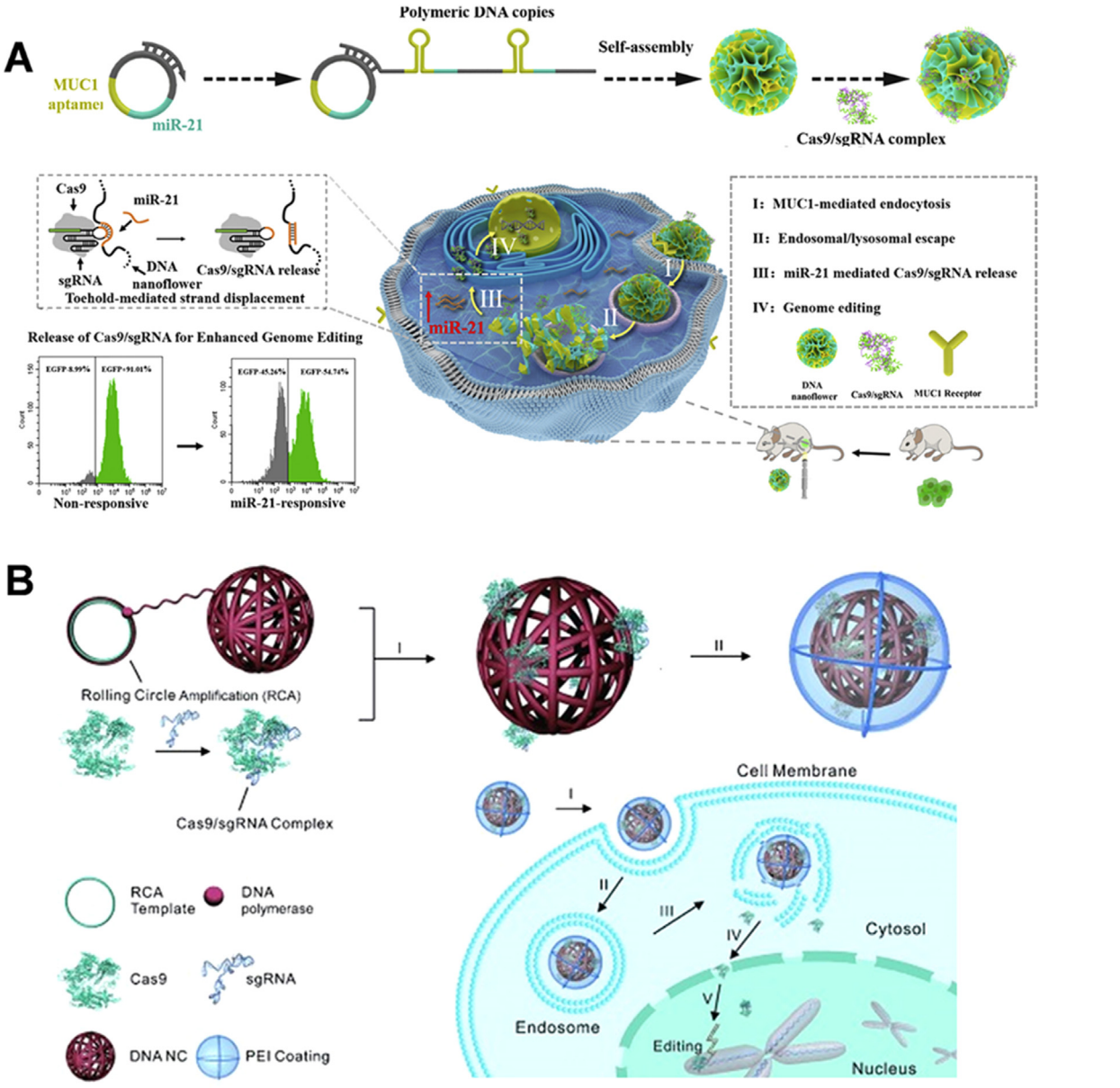

Fig. 8 – Schematic diagram of DNA nanostructures delivery of CRISPR/Cas9 system. (A) Schematic diagram of the preparation of microRNA-responsive DNF and its genome editing. Cas9/sgRNA is loaded onto DNF by sequence hybridization and endocytosed into the cell mediated by Mucin 1 (MUC1) aptamers, followed by Cas9/sgRNA release and genome editing in the nucleus. Adapted with the permission from [122]. Copyright 2020. Elsevier. (B) Schematic diagram of the process of synthesizing yarn-like DNA nanoclew by rolling circle amplification and delivery of Cas9/sgRNA to the nucleus for genome editing. Adapted with the permission from [125]. Copyright 2015. John Wiley and Sons.

Due to their complex origins and the variations observed among individual patients, achieving a complete cure for cancer remains challenging. However, with the advent of genomic exploration and the advancements in molecular biology techniques, novel avenues for cancer treatment are emerging. In addition to conventional therapeutic approaches like surgery, radiotherapy, chemotherapy and immunotherapy, harnessing the power of CRISPR/Cas9 gene editing for tumor cells and immune cells holds great promise in pinpointing the specific genetic mutations underlying cancer and elucidating their mechanisms. This genetic precision provided by CRISPR/Cas9 offers a fresh perspective and a potential new strategy for the effective treatment of tumors (Table 3).

At present, CRISPR/Cas9 technology has achieved a significant therapeutic effect in many kinds of cancers, such as lung cancer [127], gastric cancer [128], breast cancer [129], cervical cancer [130], and so on. There are a number of CRISPR/Cas9 technology-mediated studies have entered clinical trials (Table 4). CRISPR/Cas9 technology offers promising avenues for tumor treatment. It can be utilized in two key ways: (1) gene-editing of cancer cells,

Table 3 – Summary of CRISPR/Cas9 technology-mediated human disease therapy.

Applications	Diseases	Target genes	Cells/organisms	Deliver vehicles	CRISPR/Cas9 systems	Efficiency	Ref.
In vivo	Cervical cancer	HPV16-E7	HPV16-positive cells	Polymer nanoparticles	Cas9/sgRNA plasmid	Reduce the expression of HPV16 E7 protein.	[221]
In vivo		HIF-1α	Human pancreatic cancer cell line BxPC-3 and L02 cell line	Cationic liposome	Cas9/sgRNA plasmid	Down-regulated HIF-1 α and its downstream molecules vascular endothelial growth factor and matrix metalloproteinase-9.	[222]
In vivo		KSP gene	Huh7 HCC cells	Lentivirus	Cas9/sgRNA plasmid	Disrupt the KSP gene in tumors and induce apoptosis of tumor cells.	[56]
In vivo	Hepatocellular carcinoma	EGFR	HepG2 cells	pX458	Cas9/sgRNA plasmid	The gene-chemotherapy dual delivery system effectively inhibits the expression of EGFR and its downstream PIK3-Akt pathway.	[223]
In vivo	Non-small-cell lung cancer	PD-1 gene	T cells	pST1374-Cas9	Cas9/sgRNA plasmid	Enhance the lethality of T cells to tumor cells.	[143]
In vivo	Transfusion-dependent β-thalassemia sickle cell disease	BCL11A	CD34+ hematopoietic stem and progenitor cells	Electroporation	Cas9/sgRNA complex	Downregulate BCL11A expression and increase the expression of fetal hemoglobin.	[224]
In vivo	Breast cancer	AURKA	HEK293T, MDA-MB-231, SKBR3, MCF7 cells	Lentivirus	Cas9/sgRNA plasmid	Reduced the viability of breast cancer cells and significantly inhibited tumor growth.	[176]
In vivo	Breast cancer	RLIP gene	MCF10a, MCF7 and MDA-MB231 cell lines	Lentivirus	Cas9/sgRNA plasmid	Block the RLIP gene and inhibit the proliferation of breast cancer cells.	[225]
In vivo	Colon cancer	uPAR	CRL1619 CCL247	Okayama-Berg vector	Cas9/sgRNA plasmid	Knockout of the uPAR gene leads to tumor growth inhibition, EGFR downregulation, and an increase in stemness markers.	[226]
In vivo	Colon cancer	CXCR2 IL-2	Human NK cells NK-92	dCas9-VP64 vector	Cas9/sgRNA plasmid	Up-regulate the expression of CXCR2 and IL-2, promote the metastasis of NK-92 cells to the tumor site, and obtain stronger cell killing and proliferation activity.	[227]
In vitro	Recessive dystrophic epidermolysis bullosa	COL7A1	RDEB cells	Electroporation	Cas9/sgRNA complex	Targeted knockdown of faulty COL7A1 exons to produce collagen VII variants.	[228]
In vitro	Breast cancer	CXCR4 CXCR7	MDA-MB-231 cells	Lentivirus	Cas9/sgRNA plasmid	The knockout of the CXCR4 and CXCR7 genes affects the binding capacity and the functions of CXCL12 inhibit the malignant progression of triple-negative breast cancer cells significantly.	[229]
In vitro	Colorectal cancer	APC KRAS	Colorectal cancer cell	Phenylboronic dendrimer	Cas9/sgRNA complex	Significantly inhibit tumor growth and greatly prevent colorectal cancer-induced liver metastasis and lung metastasis.	[138]

(*continued on next page*)

Table 3 (*continued*)

Applications	Diseases	Target genes	Cells/organisms	Deliver vehicles	CRISPR/Cas9 systems	Efficiency	Ref.
In vitro	Ovarian cancer	EGFL6	SKOV3	Lentivirus	Cas9/sgRNA plasmid	Knockout of EGFL6 significantly inhibited tumor angiogenesis and promoted tumor cell apoptosis.	[230]
In vitro	Gastric cancer	NEAT1	Human gastric cancer cell line	pX459	Cas9/sgRNA plasmid	Downregulation of NEAT1 inhibits tumor cell growth, cell cycle progression, and metastasis, and increases apoptosis.	[128]
In vitro	Gastric cancer	PDEF gene	Gastric cancer tissue specimens and their corresponding peritumoral tissues	pSpCas9 (BB)−2A-Puro (pX459)	Cas9/sgRNA plasmid	Knockout of the PDEF gene inhibited the proliferation, migration, and invasion of gastric cancer cells.	[231]
In vitro	GBM	TIM3	NK cell	Electroporation	Cas9/sgRNA complex	TIM3 knockout enhanced NK cell-mediated growth inhibition of GBM cells.	[144]

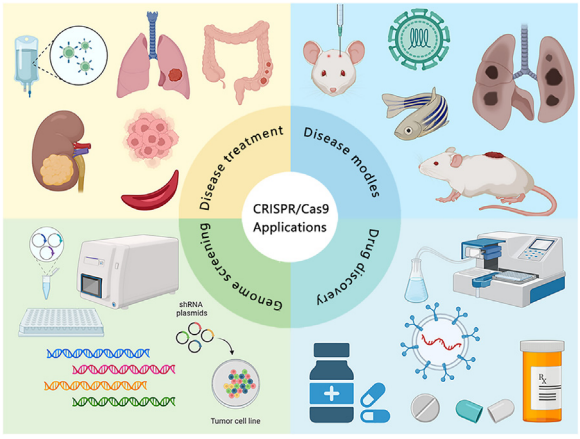

Fig. 9 – Schematic diagram of the biomedical applications of CRISPR/Cas9 technology in various fields.

enabling the killing of tumors and selectively knocking out oncogenes to impede tumor progression; and (2) improving the efficacy of engineered T cells for immunotherapy, empowering them to effectively target and attack tumors. Gene editing of cancer cells provides a more direct approach to impede the progression and metastasis of tumors [131,132]. Cervical cancer, predominantly caused by high-risk human papillomavirus (HPV) infection, poses a grave health concern for a significant number of women [133,134]. Through meticulous screening and validation of cervical cancer oncogenes, researchers have identified a significant association with the E7 oncogene in HPV-induced carcinogenesis. In light of this discovery, Gao et al. [130] utilized CRISPR/Cas9 editing technology to effectively eliminate the HPV E7 oncogene and selectively suppress its expression in cervical cancer cells, thereby presenting a potential treatment for HPV-associated cervical cancer (Fig. 10A). Chemotherapy resistance has posed a substantial obstacle to achieving optimal outcomes in cervical cancer treatment. To address this challenge, Li's group [135] devised a novel strategy by combining the chemotherapeutic drug docetaxel (DOC) with a gene therapy approach. They employed CRISPR/Cas9 packaged within a cationic liposomal 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) vector to knock out key oncogenes E6 and E7 in cervical cancer cells. This intervention successfully reactivated the p53 and pRb oncogenic pathways and induced apoptosis in cancer cells, enhancing the efficacy against chemotherapy-resistant cervical cancer. Overexpression of the Zeste homolog 2 (EZH2) oncogene has been frequently observed in various cancers, including prostate cancer. Hence, for the treatment of prostate cancer, a promising therapeutic approach involves the targeted knockdown of EZH2 using the CRISPR/Cas9 system. The successful integration of gene therapy and chemotherapy holds great promise in overcoming chemotherapy resistance, a significant challenge in cancer treatment. This combination approach not only provides a valuable solution for addressing resistance problems but also serves as a reference point for the development of individualized treatment strategies for other types of cancer. In the realm of cancer treatment, the development of low-toxicity inert platinum (IV) precursors has opened new possibilities [136]. Taking advantage of this advancement, Zhang et al. [137] devised a chain-breaking platinum (IV) backbone polymer nanoplatforms (NPCSPt) for the delivery of an EZH2-targeted CRISPR/Cas9 system (NPCSPt/pEZH2), creating a synergistic approach for the treatment of human prostate cancer (Fig. 10B). This innovative strategy combines chemotherapy and gene therapy for enhanced therapeutic

Table 4 – Clinical trials of the CRISPR/Cas system for cancer therapy. Date from https://Clinicaltrals.gov/ (last updated 16/08/2023).

NCT No.	Conditions or diseases	Interventions/treatments	Phases	First posted time
NCT04438083	Renal Cell Carcinoma	Biological: CTX130	I	2020.06.18
NCT03057912	HPV-related Cervical; Intraepithelial NeoplasiaI.	Biological: TALEN; Biological: CRISPR/Cas9.	I	2017.02.20
NCT03525652	Prostate Cancer	Biological: Therapeutic vaccine; Biological: PD-1 Knockout T Cells.	I/II	2018.05.16
NCT04426669	Metastatic Gastrointestinal Cancers	Drug: Cyclophosphamide; Drug: Fludarabine; Biological: Tumor-Infiltrating; Lymphocytes (TIL); Drug: Aldesleukin.	I/II	2020.06.11
NCT02793856	Metastatic Non-small Cell Lung Cancer	Drug: Cyclophosphamide; Other: PD-1 Knockout T Cells.	I	2016.06.08
NCT03081715	Esophageal Cancer	Other: PD-1 Knockout T Cells	–	2017.03.16
NCT04976218	Solid Tumor	Biological: TGFβR-KO CAR-EGFR T Cells	I	2021.07.26
NCT04417764	Advanced Hepatocellular Carcinoma	Procedure: Transcatheter arterial chemoembolization; Biological: PD-1 knockout engineered T cells.	I	2020.06.05
NCT03606486	High-Grade Ovarian Serous Adenocarcinoma	Other: Biospecimen Collection; Other: Laboratory Biomarker Analysis; Device: Lavage; Other: Pap Smear.	Not Applicable	2018.07.30
NCT02863913	Invasive Bladder Cancer Stage IV	Biological: PD-1 Knockout T Cells; Drug: Cyclophosphamide; Drug: IL-2.	I	2016.08.11
NCT04502446	T Cell Lymphoma	Biological: CTX130	I	2020.08.06
NCT03399448	Multiple Myeloma	Biological: NY-ESO-1 redirected autologous T cells with CRISPR-edited endogenous TCR and PD-1; Drug: Cyclophosphamide; Drug: Fludarabine; Device: NY-ESO-1 expression testing.	I	2018.09.05
NCT04557436	B Acute Lymphoblastic Leukemia	Drug: PBLTT52CAR19	I	2020.09.21
NCT05037669	Acute Lymphoblastic Leukemia; Chronic Lymphocytic Leukemia; Non Hodgkin Lymphoma.	Biological: PACE CART19	I	2021.09.08
NCT04767308	CD5+ Relapsed/Refractory Hematopoietic Malignancies	Biological: CT125A cells; Drug: Cyclophosphamide, fludarabine.	I	2021.02.23
NCT03166878	B Cell Leukemia; B Cell Lymphoma.	Biological: UCART019	I/II	2017.05.25

outcomes. Furthermore, there have been notable examples of simultaneous knockdown of multiple genes using sgRNAs with distinct sequences. Researchers discovered a strong synergistic promotion of adenomatous polyposis coli (APC) and KRAS mutations in human colorectal carcinogenesis. In light of this finding, Wan et al. [138] presented a delivery strategy utilizing hyaluronic acid-modified phenylboronic dendrimers to effectively target the Cas9 RNP for APC and KRAS mutations. Through this approach, a double-stranded genome editing system was designed with two sgRNA sequences to specifically target the mutated APC and KRAS genomic loci. This led to efficient knockdown of APC and KRAS, resulting in significant inhibition of tumor cell growth and notable prevention of colorectal cancer-induced liver metastasis and lung metastasis (Fig. 10C). Apart from suppressing oncogenes, inhibiting pathways that promote tumor cell growth is another effective approach to impede tumor progression. Researchers have discovered that the kinesin spindle protein (KSP) plays a crucial role in spindle assembly and centrosome separation during mitosis. Inactivation of the KSP protein leads to cell cycle arrest during mitosis [139]. Taking advantage of this knowledge, Lee et al. [56] utilized the functional properties of the KSP protein in their study. They introduced CRISPR/Cas9 recombinant plasmids, packaged into hepatitis C virus/E1E2-pseudotyped LV vectors, and injected them into hepatocytes for targeted delivery to tumor sites. This targeted delivery allowed for the disruption of the KSP gene in hepatocellular carcinoma

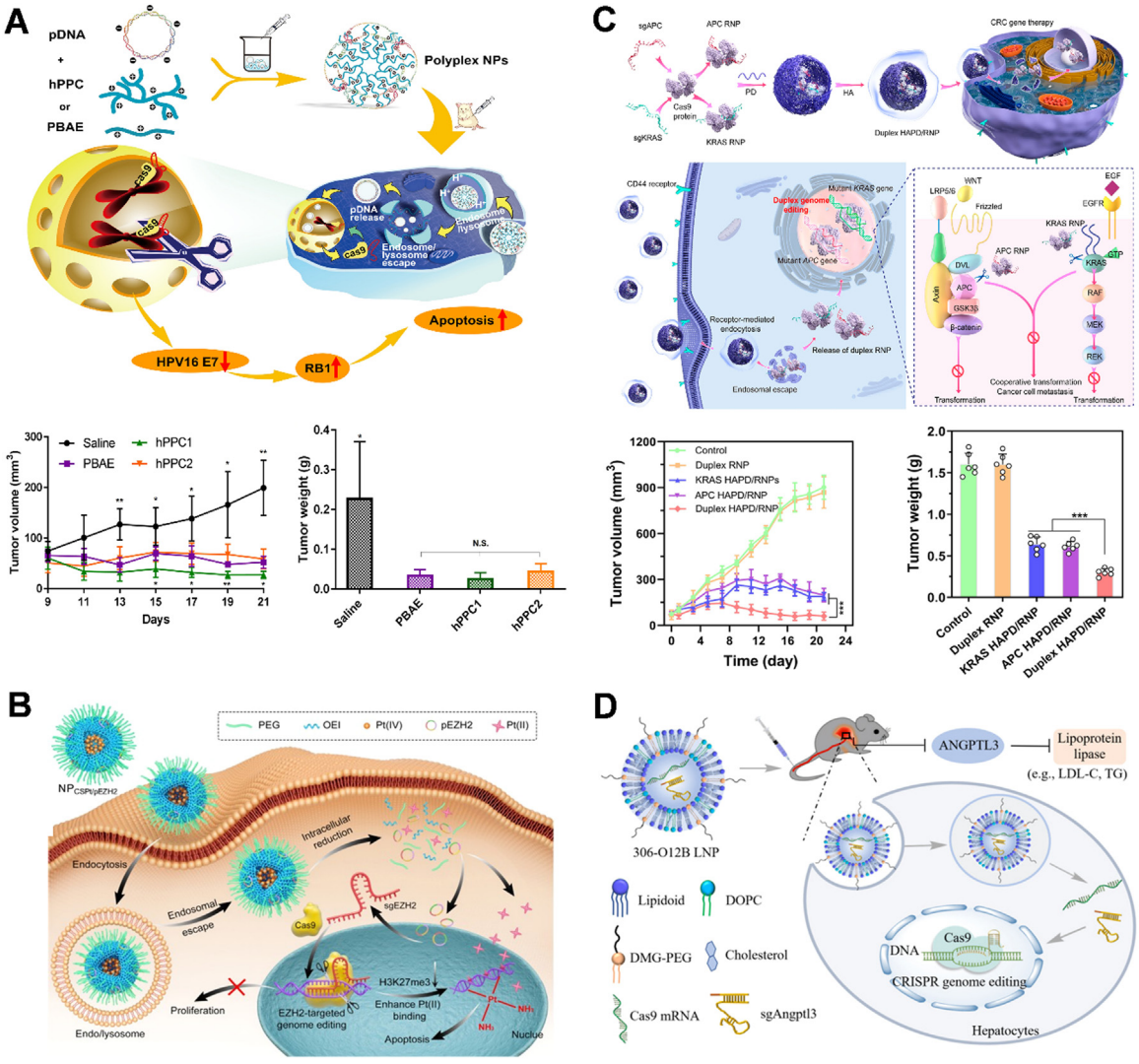

Fig. 10 – Schematic diagram of CRISPR/Cas9 system for human disease treatment. (A) Preparation of poly(amide-amine)-poly(β-amino ester) hyperbranched copolymer (hPPCs)/linear poly(β-amino ester) (PBAE)–16E7t1 polymeric nanoparticles and the mechanism and *in vivo* antitumor effect for the treatment of HPV infection-associated cervical cancer. Adapted with the permission from [212]. Copyright 2020. Elsevier. (B) Schematic diagram of the chain-shattering Pt (IV)-backboned polymeric nanoplatforms to deliver EZH2-targeted CRISPR/Cas9 system (NPCSPt/pEZH2) for effective gene editing-chemo synergistic treatment of prostate cancer. Adapted with the permission from [137]. Copyright 2020. Springer Nature. (C) Preparation of hyaluronic acid-decorated phenylboronic dendrimer (HAPD)/ RNP nanoparticles and activation of Wnt/β-catenin and RAS-extracellular signal-regulated kinase (RAS-ERK) signaling pathways and *in vivo* antitumor effects in the treatment of colorectal cancer. Adapted with the permission from [138]. Copyright 2021. American Chemical Society. (D) Schematic diagram of LNP-mediated CRISPR-Cas9-based *in vivo* genome editing to induce Angptl3 loss of function and reduced LDL and triglyceride levels. Adapted with the permission from [158]. Copyright 2021. National Academy of Sciences.

(HCC) cells. Consequently, the cells experienced mitotic cell cycle arrest and apoptosis induction, effectively inhibiting tumor growth. CRISPR-based gene activation (CRISPRa) is a promising gene therapy approach that selectively targets promoters or enhancers in a tissue/cell type-specific manner to upregulate gene expression [140]. In HCC, epigenetic silencing of tumor suppressor genes (TSGs) is a crucial characteristic. Sgro et al. [141] developed a customizable epigenetic editing toolkit using epigenetic effector domains and gRNA for CRISPRa, which can target multiple candidate TSGs in HCC cells. This toolkit can be used to investigate the multifaceted roles of silenced TSGs and holds potential as a precise oncological tool for treating liver cancer.

Additionally, besides directly targeting cancer cells through gene editing, an alternative strategy involves manipulating T cells, which play crucial roles in facilitating cytotoxicity, generating immune memory, and enhancing humoral immunity in the context of anti-tumor responses. Recognizing the pivotal role of T cells in immunotherapy, especially in the realm of immune checkpoint inhibitors and

chimeric antigen receptor T cell therapies, scientists have directed their efforts towards harnessing the therapeutic potential of T cells [142]. Exploiting the CRISPR/Cas9 system, numerous tumor immunotherapies have been developed. Noteworthy among them is the rapid advancement of the chimeric antigen receptor concept over the past thirty years, which holds immense promise for revolutionizing tumor immunotherapy. A significant milestone was achieved by Lu et al. [143] through the pioneering of a phase I clinical trial involving CRISPR/Cas9-edited T cells that specifically targeted the PD-1 gene in patients diagnosed with advanced non-small cell lung cancer (NSCLC). To enhance the ability of T cells to kill NSCLC tumor cells, researchers employed a strategy involving the design of a pair of sgRNAs to disrupt the PD-1 gene in T cells. Plasmids containing Cas9/sgRNAs were then introduced into T cells using electroporation. This method aimed to enhance the anti-tumor activity of T cells. In the context of GBM, a primary malignant brain tumor, the role of Natural Killer (NK) cells is crucial. NK cells are highly effective cytotoxic cells that combat GBM cells induced by tumors. Notably, the expression of T cell immunoglobulin mucin family member 3 (TIM3) on NK cells is considered an indicator of NK cell dysfunction. Building upon this knowledge, Morimoto et al. [144] successfully suppressed the growth of GBM cell lines by utilizing CRISPR-Cas9 and electroporation techniques to knock down TIM3 in primary human NK cells. This groundbreaking experiment represents the first documented case of TIM3 knockdown achieved through electroporation in NK cells derived from healthy peripheral blood. The potential of NK cell-based immunotherapy as an innovative approach for GBM treatment, coupled with the inhibition of PD-1 through checkpoint receptor blockade, holds tremendous promise. With the progress of medical research in the field of genetic diseases, the use of CRISPR/Cas9 technology presents a tremendous opportunity for treating a wide range of neurological disorders. Specifically, in recent years, notable advancements have been achieved in utilizing CRISPR/Cas9 technology to address Huntington's disease, building upon the extensive body of previous research. Several reports have demonstrated the remarkable clinical effectiveness of CRISPR/Cas9 technology in intervening and treating Huntington's disease [145]. Additionally, the application of CRISPR/Cas9 gene-editing technology is steadily expanding to encompass etiological research, treatment, and intervention of neurodegenerative disorders such as AD [146]. While challenges still exist for the CRISPR/Cas9-based treatment of AD, the development of various engineered disease models has offered valuable insights. Furthermore, the role of CRISPR/Cas9 technology in targeting disease-causing genes and associated factors in neurological disorders has been extensively examined and verified. The field of medical research focused on utilizing the CRISPR/Cas9 system for gene editing therapies is experiencing rapid growth. It is increasingly recognized for its prominent role in addressing diseases with complex etiologies like DMD [147], liver diseases [148], and retinal diseases [80]. DMD is a genetic muscle disease caused by mutations in the DMD gene located on the X chromosome [149,150]. Currently, there are two approved therapies for DMD: steroid supplementation and antisense oligonucleotide (AON)-based approaches. While these treatments offer some relief from DMD symptoms, they have limitations such as side effects and limited efficacy [151]. Conversely, CRISPR/Cas9 technology holds promise as a potential solution for permanent correction of genetic diseases [9]. Xiao et al. [152] introduced an *in situ* genetic correction method using CRISPR/Cas9 that involved precisely adding the missing exon in patient-derived induced pluripotent stem cells (iPSCs). This approach successfully rebuilt the complete coding sequence for the dystrophin protein in iPSCs derived from DMD patients with an exon 50 deletion. The expression and localization of dystrophin protein were restored in the genetically corrected iPSCs and their derived cardiac muscle cells. These findings demonstrate the clinical potential of this *in situ* gene correction strategy for DMD gene therapy, particularly in conjunction with advancements in cell transplantation therapies. Wilson's disease is an autosomal recessive monogenic liver disease caused by dysfunction of the ATPase copper transporter β (ATP7B) [148]. Wei et al. [153] initially generated ATP7B cells with point mutations using the CRISPR/Cas9 system. Subsequently, they employed CRISPR/Cas9 technology along with specific single-stranded oligo-DNA nucleotides to genetically correct these point mutations. The study successfully demonstrated the efficiency of CRISPR/Cas9 in introducing specific ATP7B mutations and correcting ATP7B point mutations, establishing the first instance of genetic correction of an ATP7B point mutation at the human cellular level. These findings indicate the feasibility of CRISPR/Cas9-mediated correction for ATP7B point mutations and suggest its potential for clinical translation. Damage or loss of the neurosensory retina is ultimately caused by dysfunction of the retinal pigment epithelium (RPE) [154]. CRISPR interference (CRISPRi) is a technique that employs a Cas9 enzyme without nuclease activity, combined with transcriptional repressors, to modulate the expression levels of endogenous genes [155]. Gilbert et al. developed a CRISPRi system for human RPE cells utilizing an inactive SpCas9 (dCas9) fused with the Kruppel-associated box transcriptional repressor domain. This system can be directed by sgRNA to induce specific gene repression [156]. By specifically targeting and repressing the high expression of TMEM97 in RPE cells, the study demonstrated the functional role of TMEM97 in regulating oxidative stress and RPE degeneration. This CRISPRi system provides a valuable tool for investigating the functional implications of genetic factors involved in RPE dysfunction associated with age-related macular degeneration. Treating diseases by rectifying pathogenic gene mutations is a viable strategy. Vascular endothelial growth factor-A (VEGF-A) plays a pivotal role in the proliferation and permeability of vascular cells. It is intricately associated with retinal blood vessels in prevalent retinal vascular disorders like diabetic retinopathy and retinal vein occlusion. The standard therapeutic approach for these conditions involves the intravitreal administration of anti-vascular growth factor medications such as bevacizumab and ranibizumab. This form of treatment aims to reduce the levels of VEGF-A protein and inhibit its proliferation within retinal blood vessels. However, these drugs require frequent and long-term injections, leading to poor patient compliance and potential side effects like intraocular inflammation.

CRISPR/Cas9 technology offers promising therapeutic avenues for addressing such diseases. Ameri and colleagues [157] utilized liposomes as carriers to deliver CRISPR-Cas9 RNP complexes for targeting Muller and RPE cells, which are the primary producers of VEGF-A protein. The objective was to disrupt the VEGF-A gene, leading to a significant decrease in VEGF-A protein levels. Remarkably, they observed a reduction of 43% in VEGF-A protein levels in RPE cells, and a reduction of 38% in Muller cells, as compared to untreated cells. The researchers emphasized the advantageous use of liposomes as RNP vectors in this particular study. Liposomes possess non-targeting characteristics, allowing gene editing to occur across multiple cells rather than being limited to a specific cell type, making them more suitable for diseases requiring gene editing in multiple cell types compared to specifically targeted vectors like LV. Furthermore, in the management of lipoprotein metabolism disorders in humans, the focus on angiopoietin-like 3 (Angptl3) as a potential therapeutic target has gained significance due to its correlation with lipid levels. Expanding on this concept, Qiu et al. [158] developed liposomal nanoparticles capable of delivering Cas9 mRNA and sgRNA to achieve Angptl3 knockdown and lower levels of low-density lipoprotein (LDL) and triglycerides. Notably, the liposomal nanoparticles exhibited significantly higher delivery efficiency compared to the gold standard MC-3 LNP for liver-targeted nucleic acid delivery (Fig. 10D).

4.2. *Construction of disease models*

Disease occurrence involves intricate pathological processes, making it impractical to use the human body directly for studying disease mechanisms and assessing drug effectiveness. Consequently, the establishment of animal models becomes crucial. Building animal disease models is an essential and indispensable component of exploring the mechanisms underlying human diseases. These models contribute valuable insights into genetics, pathology, and physiology. The advent of CRISPR/Cas9 editing has unlocked significant potential for modeling genetic variations in animal models, providing a powerful tool for *in vitro* research.

The most commonly used animal model for studying diseases is mice, primarily due to their ease of breeding and rapid reproduction. CRISPR/Cas9 technology has enabled the construction of disease models in mice. For instance, a mouse model of Musculocontractural Ehlers-Danlos syndrome (mcEDS) was created by directly injecting a plasmid expressing CRISPR/Cas9 into the prokaryotic nucleus of single-cell embryos using a pX330 vector. This approach targeted the knockout of Chst14, the causative gene of mcEDS, and successfully overcame the high perinatal lethality observed in conventional methods [159]. In addition, simpler and more efficient methods for constructing mouse models have been developed. Ishibashi et al. [160] introduced the SUCCESS (Single-strand oligodeoxynucleotides, Universal Cassette, and CRISPR/Cas9 produce Easy Simple knock-out System) method, which allows for gene knockout in mouse tumor cell lines. This approach does not require constructing a target vector and can be widely employed to generate homozygous knock-out cell lines and knock-in cell lines. However, in the case of neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease, although mouse disease models have been extensively used to investigate their pathogenesis, primate large animal models have proven more adept at reproducing the pathological features of these neurodegenerative diseases than rodent models like mice. This is largely due to the divergent brain development between primates and rodents, resulting in significant differences in neural circuits, lifespan, and aging time between the two groups [161]. Another study further demonstrated that nonhuman primates possess physiological characteristics highly similar to humans, making them an ideal choice for constructing human disease models. Wang and colleagues conducted a study where they utilized CRISPR/Cas9 technology to disrupt the X-linked dystrophin gene in monkeys, effectively creating a model for DMD. Through whole-genome sequencing analysis, they found no off-target effects on the target genes in the monkey disease model, unlike what has been observed in rodent models [162]. This experiment indicated that CRISPR/Cas9 technology may offer a safer and more effective approach for constructing disease models in nonhuman primates, presenting promising opportunities for therapeutic research on human diseases. This finding also helps alleviate concerns regarding safety issues, such as the potential off-target effects associated with CRISPR/Cas9-mediated transgenic animal models.

Many diseases pose challenges in achieving targeted molecular intervention therapies due to the absence of appropriate animal disease models. One such disease is retinoblastoma, which has a high incidence in children aged 3 years and younger. Despite improvements in the cure rate, there is a lack of molecular-level disease models to identify potential therapeutic targets. However, Naert et al. [163] addressed this issue by constructing a tropical African Xenopus retinoblastoma model using CRISPR/Cas9-mediated knockdown of rb1 and rbl1 genes. This approach allowed for the rapid identification of crucial targets for therapeutic intervention, marking a significant advancement in retinoblastoma treatment. By employing CRISPR/Cas9 technology, the model construction process becomes more precise and efficient, bypassing the laborious procedures involved in deriving embryonic stem cells or genetic hybridization [164]. Consequently, CRISPR/Cas9's involvement in this research contributes to the progress of medical research by facilitating the development of disease models and accelerating discoveries in targeted therapies.

4.3. *Genome screening and drug discovery*

The causes of many diseases are complex, particularly those that are associated with genetic factors. Therefore, studying their pathogenesis at the molecular level of DNA is crucial. However, analyzing gene function and screening pathogenic genes in animal models have long been a challenging task. Fortunately, the advent of CRISPR/Cas9 technology has provided a promising solution to this problem, particularly when it comes to screening hundreds of candidate genes. This revolutionary technology has brought new opportunities for researchers to investigate gene function

and identify pathogenic genes accurately. Using CRISPR/Cas9 technology, the first step involves designing and synthesizing a whole-genome mutation library or a gene mutation library associated with specific functions. After transfecting the gRNA library into cells, CRISPR/Cas9-mediated gene editing is responsible for identifying and cleaving specific target genes. Finally, the cell population is screened and analyzed to identify and validate genes related to the biological processes, diseases, or drug responses of interest. This entire process is referred to as CRISPR/Cas9 gRNA library screening technology [165,166]. Functional genome screening using CRISPR can reveal phenotypic changes after drug treatment or other stimuli, thus identifying new targets for cancer therapy [167]. So far, CRISPR libraries have been employed in various studies, including the screening of genes associated with congenital heart diseases [168], HCC suppressor genes [169], and cancer initiation and progression [170]. The experimental approach utilized by Yamamoto et al. using CRISPR library technology effectively identifies the molecular factor M1AP as an activator of the key oncogene MYC. This finding is of significant importance in elucidating the regulatory mechanisms of MYC expression and furthering drug research targeted at malignant tumors [171]. HAN's research group carried out genome-wide CRISPR/Cas9 gene knockout screening on human lung epithelial cells and found capsicum (CIC), an important immunomodulatory factor in influenza A virus (IAVs) infection, which successfully solved the problems of seasonal vaccine inefficacy and drug resistance [172]. Huang et al. [173] conducted an optimization of screening conditions and strategies to identify candidate gRNA for HIV proviruses in astrocytes. Their study not only provides valuable insights for other high-throughput screening studies but also sheds light on the importance of genome screening. It not only unravels the molecular-level pathological mechanisms but also serves as an early preparatory stage and essential link in new drug research and development.

The advent of the CRISPR/Cas9 technique has ushered in a groundbreaking transformation in the realm of drug discovery and development. It has significantly impacted various aspects such as drug target discovery and drug screening. The rapid advancement of computer-aided drug design and diverse gene editing technologies has accelerated the progress of new drug research and development strategies. Through genetic engineering, specific genes responsible for certain diseases can be identified and utilized as drug targets for the development and screening of relevant new drugs [174]. CRISPR/Cas9 technology enables the swift screening of potential target genes. As a result, numerous disease-associated causal genes have been identified, leading to the development of corresponding drugs for disease treatment. As an example, Imatinib was formulated as a precise therapeutic agent to specifically target and address the pathology of chronic myeloid leukemia triggered by the fusion of BCR-ABL genes. Later on, vemurafenib was discovered through screening, which showed that mutations in BRAF V600E are responsible for melanoma [175]. There have been notable instances where the exploration of drug combinations in breast cancer therapy has been noteworthy. For example, the overexpression of Aurora-A in breast cancer has been associated with a poor prognosis. Although MLN8237, a small molecule kinase inhibitor of Aurora-A, is commonly employed for breast cancer treatment, its efficacy is limited. To address this issue, Chen et al. [176] conducted a systematic screening using the CRISPR-Cas9 gene knockout library targeting 507 kinases. Their objective was to identify a lethal target that could enhance the synthesis of MLN8237 in breast cancer cells. Through their study, they discovered that combining MLN8237 with the Haspin Kinase inhibitor CHR-6494 exhibited a synergistic effect. This resulted in reduced viability of breast cancer cells and substantial inhibition of tumor growth. This research highlights the potential of combining different drugs to enhance their effectiveness in the treatment of breast cancer. Furthermore, drug resistance is a significant concern in the clinical use of various medications. However, CRISPR/Cas9 technology can assume a critical role in identifying potential targets for drug resistance and reversing genes responsible for this resistance. This has profound implications in the early stages of clinical drug development, enabling the formulation of strategies to overcome drug resistance and improve treatment outcomes. Shen et al. [177] employed the quantitative trait locus (QTL) mapping method and the CRISPR/Cas9 technique to map the drug resistance gene TgME49_290,860 in Toxoplasma gondii. The findings indicated that, in comparison to QTL mapping, the CRISPR/Cas9 gene-editing technique exhibited slightly higher efficiency in the identification of drug-resistance genes. Numerous studies have reported the efficacy of FGFR inhibitors in the treatment of gastric cancer. However, it is observed that cancer cells can develop resistance to FGFR inhibitors, compromising their sensitivity. In addressing this challenge, several studies have conducted screenings of diverse kinases. Ultimately, the kinase ILK was employed to enhance the inhibition of FGFR, effectively overcoming the issue of drug resistance [178].

5. Challenges and perspectives

Although CRISPR/Cas9 technology has made substantial strides in the field of gene therapy, the effective and secure delivery of this technology still presents significant challenges. At present, three primary barriers impede the advancement of CRISPR/Cas9 technology: off-target effects targeted delivery efficiency, and immunogenicity. Addressing these challenges requires continued investigation and refinement.

5.1. *Off-target effects*

The issue of off-target effects presents a significant hurdle for the successful implementation of CRISPR/Cas9 technology in gene editing endeavors. These effects occur when Cas9 proteins bind to PAM-like sequences or when guide RNAs (gRNAs) bind to unintended nucleotide sequences at the target site, and their reported efficiency exceeds 50% [179]. Off-target cleavage can result in undesirable mutations and cytotoxicity, potentially disrupting the function of normal genes. Addressing off-target effects involves optimizing three key aspects: sgRNA selection and design, Cas9 protein optimization, and overall CRISPR/Cas9 system optimization.

To begin with, the specificity and efficacy of sgRNA are crucial factors in gene editing using the CRISPR/Cas9 system. Therefore, the selection and design of an optimal sgRNA sequence are vital for minimizing or eliminating off-target effects. Research has indicated that the structure of sgRNA influences its targeting capability, and an increased GC content at the PAM (Protospacer Adjacent Motif) end of the sgRNA sequence improves its targeting efficiency [180]. Additionally, shortening the length of sgRNA sequences has been demonstrated to enhance their sensitivity to mismatches with residual target DNA, leading to a significant decrease in off-target effects, by up to approximately 5000-fold [181]. Another approach to mitigate off-target effects involves chemically modifying sgRNAs using bridging or locked nucleic acids, which has shown promising outcomes in reducing off-target effects [182]. It is crucial to predict potential off-target cleavage events caused by specific sgRNAs before initiating the gene editing process. Various methods have been developed for the detection of off-target sgRNAs, including the T7E1 endonuclease assay [183], genome-wide off-target analysis by two-cell embryo injection [184], primer extension-mediated sequencing [185], chromatin immunoprecipitation followed by sequencing [186], and verification of *in vivo* off-targets [187], etc.

In the context of enhancing the Cas9 protein, the development of high-fidelity SpCas9 mutants is regarded as the most promising strategy to address off-target effects [188]. Several spCas9 variants have been reported, such as SpCas9-NG [189], xCas9 [190], evoCas9 [191], and SpCas9-HF1 [188], among others. These variants exhibit improved fidelity compared to SpCas9 and can substantially reduce the occurrence of off-target effects. Once the spCas9 variant is generated, the subsequent challenge involves selecting the most suitable variant for a specific target sequence [192] that can also inactivate the Cas9 protein promptly. Replacing plasmids with RNPs not only ensures higher specificity but also avoids the problem of continuous Cas9 protein expression.

DSBs in DNA are typically repaired through two primary pathways: HDR and NHEJ. While NHEJ is the predominant DSB repair mechanism in most mammals, it is error-prone and often results in the insertion or deletion of genetic materials. Conversely, HDR enables precise genomic repair but some cells, especially human cells, exhibit a preference for NHEJ during DSB repair. Consequently, reducing or eliminating DSBs at non-target sites and enhancing the efficiency of HDR are important objectives. To shift the balance of DSB repair from NHEJ to HDR, the inhibition of the NHEJ pathway can be achieved through various methods. This includes using small-molecule inhibitors targeted at NHEJ, inhibiting key NHEJ factors, or employing activators of HDR. Another approach involves promoting the HDR process by extending the S and G2 phases of the cell cycle, which facilitates HDR. These strategies aim to enhance the efficiency of gene editing and improve the accuracy of DSB repair. Furthermore, there have been significant advancements in gene editing technology. Two notable editing tools are cytosine base editors (CBEs) and adenine base editors (ABEs), which have been developed to directly introduce point mutations without relying on DSBs or subsequent repair processes [193]. These base editors have demonstrated the ability to greatly enhance gene editing efficiency while reducing off-target effects. Consequently, they represent a promising technology for improving editing efficiency.

5.2. *Delivery efficiency*

Prolonged exposure to viral vectors after their delivery into the body poses risks such as mutation, off-target cleavage, and potential immune responses *in vivo* [194]. To address these concerns, plasmids can be replaced with mRNA in the form of RNP, which reduces the residence time of viral vectors in the host, inhibits off-target activity, and increases efficacy. However, non-viral vectors still encounter several challenges. These include insufficient accumulation in target tissues, phagocytic clearance by the organism during delivery, degradation by proteases or nucleases, cytoplasmic migration, and difficulties in nuclear delivery [23]. Firstly, researchers have dedicated numerous studies to improve the accumulation of non-viral carriers at the target site. One approach involves leveraging the enhanced permeability and retention (EPR) effects to promote the aggregation of nanocarriers into tumors [195]. Additionally, physical exogenous factors such as radiation, ultrasound, thermotherapy, and photodynamic therapy can be applied to enhance the EPR effect [196]. Secondly, the development of targeted and smart non-viral vectors, such as incorporating aptamer modifications on their surface for active targeting, is crucial for enhancing their accumulation at the desired target site. NLSs can be recognized by the nuclear import receptor Importin α, initiating the transport of cargo from the cytoplasm to the nucleus [197]. Therefore, incorporating NLSs into vectors can facilitate the efficient delivery of cargo to the nucleus [117,198].

5.3. *Immunogenicity*

The immune response elicited by the Cas9 protein or delivery vectors in the host presents an additional obstacle in the application of CRISPR/Cas9 technology. The Cas9 protein, derived from Streptococcus pyogenes, is considered an exogenous antigen that can potentially induce an immune response upon introduction into the body. Research has shown a pre-existing immune response to homologous Cas9 proteins in SaCas9 [199], which amplifies the immune response mechanism upon Cas9 protein activation. Nonetheless, multiple strategies can be employed to address the generation of an immune response against the Cas9 protein. Firstly, gene editing using CRISPR/Cas9 technology in the early stages of life can be utilized to treat childhood genetic diseases or even screen for potential diseases during fetal development, as the immune system is not fully developed at that time. Secondly, immune-privileged organs such as the eyes, brain, placenta, and testis can be targeted since Cas9 proteins do not elicit immune rejection in these organs [200]. Moreover, exploring alternative Cas9 protein variants or programmable nucleases from different species that elicit fewer immune responses is another viable option [201]. Additionally, the utilization of delivery vectors equipped with self-deletion

capability can prevent the persistent expression of Cas9 protein [202].

Furthermore, although viral vectors are effective in delivering and expressing gene constructs, their immunogenicity and the presence of pre-existing immunity in humans pose significant limitations when utilizing virus-based vectors for CRISPR/Cas9 components delivery. Prolonged exposure to viral vectors upon introduction into the human body can trigger immune responses [194]. To mitigate the risks of off-target effects and immune responses, alternative strategies have been employed, such as using RNP complexes or mRNA instead of plasmids. These alternatives shorten the duration of viral vector presence in the host. Additionally, various types of non-viral vectors, including inorganic nanoparticles, lipids, and polymers, have been investigated as alternative delivery systems for the CRISPR/Cas9 system. These non-viral vectors have demonstrated improved safety and lower immunogenicity [203]. Particularly, LNPs have been extensively utilized as tools for delivering various molecules into cells. LNPs address safety and immunogenicity concerns due to the absence of viral components. For instance, LNPs with low immunogenicity enable the repeated administration of Cas9 mRNA for gene editing in mouse skeletal muscles, offering a potential treatment for DMD [204]. Moreover, a delivery nanoplatform based on fish egg protein-functionalized AuNCs has shown excellent gene editing and bioimaging capabilities when delivering Cas9-sgRNA plasmids. Gold nanoparticles serve as a nontoxic carrier for drug and gene delivery applications, overcoming the immunogenicity associated with viral vectors [205].

Rath emphasized two risks related to the use of CRISPR/Cas9 gene editing technology, underscoring the importance of paying attention to its biosafety. The first risk pertains to humans, particularly those connected to therapeutic applications of genome editing. The second risk involves potential environmental consequences resulting from the manipulation of animals, plants, and microorganisms through genome editing techniques [206]. Consequently, it is imperative to conduct effective risk assessments and establish rigorous oversight mechanisms within the field of gene editing, not only presently but also in the future. In-depth research on these issues is crucial for achieving a more secure and efficient delivery of the CRISPR/Cas9 system.

6. Conclusions

CRISPR/Cas9 technology has brought forth a new era in the field of medical biology, offering enhanced precision and ease of manipulation compared to conventional gene editing techniques. It has brought about a revolution in research pertaining to human disease treatment, plant and animal breeding, and microbial production, with its importance continuously increasing. However, most clinical trials involving therapeutic gene editing using CRISPR/Cas9 have primarily relied on *in vitro* methods thus far. The translation of CRISPR/Cas9 towards *in vivo* gene editing presents significant challenges that need to be tackled, including concerns regarding specificity, safety, and efficient delivery. Additionally, research studies have raised apprehensions about the promotion of CRISPR/Cas9 applications, touching upon aspects such as safety, ethics, social fairness, and the risks associated with transgenic manipulation [207–210]. Consequently, it is crucial to establish a comprehensive legal framework that ensures the secure and equitable utilization of gene editing technology. The doubts and reservations surrounding this technology do not stem from the technology itself, but rather from how it is implemented and regulated. Moreover, there is an imperative to enhance the precision of CRISPR/Cas9 technology to minimize potential off-target effects. Additionally, the advancement of innovative non-viral vectors that exhibit efficiency, stability, specificity towards target cells, high payload capacity, and safety is of utmost importance to propel the clinical utilization of CRISPR/Cas9 technology and augment its efficacy *in vivo* scenarios. This holds significant relevance in addressing a broad spectrum of genetic ailments and cancer. Despite the inherent difficulties, the ongoing refinement of CRISPR/Cas9 is paving the way for its clinical implementation, enabling the potential treatment of more intricate diseases. It is anticipated that CRISPR/Cas9 will evolve into a potent instrument in the domain of gene editing, ushering in a new era of breakthroughs in biomedical research and applications.

Declaration of Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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