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# *CRISPR-Cas9 Delivery Systems and Neurological Diseases*

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***Abstract.*** Until now, the clustered regularly interspaced short palindromic repeats and their associated protein 9 gene remains the most effective gene editing system at present. It is widely used in medical clinical practice and has shown remarkable efficacy in treating diseases caused by genetic defects, such as sickle cell anemia or cancer. However, it still has shortcomings in the treatment of neurological diseases such as neurodegenerative diseases and episodic brain dysfunction. Due to the complexity of neurological diseases, choosing an appropriate delivery system is particularly important. One of the focus is the method of breaking through the blood-brain barrier and achieving neuron-targeting tissue. This article summarizes several types of delivery systems, and compare and rank the relevant data based on their delivery efficiency data in different clinical fields, such as off-target rate, immunogenicity, carrier capacity and cost. Moreover, several neurodegenerative diseases are analyzed in detail to screen out the most efficient and clinically practical delivery technologies to make conjectures about the technologies with development potential.

***Keywords:*** CRISPR-Cas9, delivery system, Alzheimer's disease, Epilepsy, Duchenne muscular dystrophy

## 1. Introduction

Neurological diseases, including neurodegenerative diseases, episodic brain dysfunction, neuromuscular diseases, etc. Due to their complex pathogenic factors, the conventional drugs on the market usually focus on symptom relief [1]. For instance, the pathological markers of neurodegenerative diseases, namely the irreversible death of neurons and the abnormal deposition of proteins. In Alzheimer's disease, the formation and deposition of amyloid-β and phosphorylated tau occur, eventually leading to extracellular amyloid plaques and neurofibrillary tangles [2]. Gene editing technologies, such as CRISPR-Cas9, can correct amyloid precursor protein (APP), Presenilin 1 (PSEN2), and Presenilin 2 (PSEN33) in the treatment of familial Alzheimer's disease (FAD) [3]. Meanwhile, for diseases with more complex pathogenic factors, CRISPR technology can also be applied as a tool to explore the pathogenic mechanism.

In terms of the mechanism of action, the essence of gene editing lies in the generation of double-strand breaks at specific genomic locations through programmable nucleases, thereby activating the cell's intrinsic DNA repair processes. This enables the targeted rewriting of genetic information, the knockout or insertion of specific DNA fragments, and ultimately achieves the objective of site-specific gene editing [4]. Gene editing technologies include transcription factor-like effector

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nuclease technology, zinc finger protein technology and CRISPR-Cas9 technology. Among them, CRISPR-Cas9 is widely used in the biomedical field due to its relatively low cost, high efficiency and easy preparation. CRISPR, clustered regularly interspaced short palindromic repeats, is an adaptive immune system in prokaryotes. By fusing crRNA and tracrRNA, a single-guide RNA (sgRNA) is formed which can target specific sequences [5]. By designing a single guide RNA (sgRNA) to form base-pair complementarity with the target DNA, it guides Cas9 to exert its endonuclease activity, acting on the target gene and causing double-strand breaks (DSBs) in the target DNA. Double-strand breaks in DNA trigger the cell's intrinsic repair mechanisms. Cells mainly repair through two competing pathways: one is non-homologous end joining, which directly connects the broken ends and often leads to small insertions or deletions, thereby achieving gene knockout; the other is precise homology-directed repair, which can use co-transferred exogenous donor DNA as a template to precisely correct genes or insert fragments, achieving more complex editing such as gene knock-in or site-directed mutagenesis. There are three main types of CRISPR-Cas9 technology in terms of the selection of delivered content [6]. The first one is delivering the plasmids encoding Cas9 protein and sgRNA. However, in the form of DNA, there is a possibility of integration into the cell genome. Moreover, it is more difficult to pass through the cell nucleus to exert its function. The second approach, delivering the mRNA encoding Ca9 protein, can solve the problems that emerged in the first approach, but mRNA is not as stable as plasmids, resulting in a shorter effective period. In addition, pre-assembled Cas9 and sgRNA complexes can be directly introduced. This approach effectively avoids the integration of exogenous DNA sequences into the genome and has low immunogenicity due to its transient effect. Moreover, in practical applications, the selection of the delivery system is also crucial in addition to the choice of contents [7].

Although physical delivery is relatively simple to implement, it is mainly applicable to extracellular delivery, such as microinjection and electroporation, directly injecting Cas9 and sgRNA into isolated cells. Polymer carriers can effectively address the issue of immunogenicity and come in various types, making them suitable for a wide range of scenarios and possessing great potential. In this review, the delivery methods of CRISPR-Cas9 from the perspectives of working principle, current development status, and applicable diseases, etc., are classified and discussed. Subsequently, several neurological diseases and current developent statues of CRISPR-Cas9 in these disease are presented. This review aims to provide a suitable delivery method which represents a promising direction for subsequent investigation.

## 2. Delivery method

### 2.1. Viral vector

#### 2.1.1. Adeno-associated virus (AAV)

AAV (adeno-associated virus) is a non-integrating vector that can express CRISPR components in a free form for a long time, thereby generating a lasting therapeutic effect. AAV shows serotype specificity when delivered to host cells, and also exhibits neurotropism [8]. It is precisely based on these properties which make AAV vectors the most widely used vectors. It has performed well in the treatment of genetic diseases such as hemophilia, Leber congenital amaurosis, and neurodegenerative diseases such as Alzheimer's disease. It is regarded as the best vector for delivering CRISPR-Cas9 in the central nervous system [9]. The main problem of AAV vectors is the induced immune response, which may lead to problems such as cell disorder and inflammatory

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responses. In terms of solutions, a special Cas9 protein can be chosen to cut the AAV vector itself, or opt for AAVs of different serotypes.

The vector capacity of AAV vectors is relatively small, only 4.7-4.8 kb, which imposes restrictions on the Cas9 nuclease gene and sgRNA they carry internally [10]. There are also many solutions. The first one is to find or modify smaller Cas9 proteins, such as using SaCas9 or Cas9's direct homologues (CjCas9, etc.). The second approach involves the Dual-AAV system. When a smaller Cas9 nuclease gene cannot be used, the CRISPR-Cas9 system are able to split into two AAV viral particles, with one carrying the Cas9 expression cassette and the other carrying the sgRNA expression cassette [11].

### 2.1.2. Adenovirus (AdV)

Adenovirus (AV), an envelop-free DNA virus, has a relatively high carrier capacity of 8kb. Due to its lack of lipid membrane, the risk of integration into the host genome is thereby reduced. However, it will still trigger an immune response and cause tissue inflammation, and then reduce the off-target rate [12]. High-capacity adenoviruses (HCAdV) and helper-dependent adenoviruses (HDAdV) are variants of adenoviruses that have gained significantly greater loading capacity than conventional adenoviruses by deleting non-essential viral genes, such as HDAdV which can reach up to 35 kb. This property enables it to carry all the components of the CRISPR-Cas9 system, achieving efficient site-specific gene integration [13].

### 2.1.3. Lentiviruses (LVs)

LVs, lentiviruses, achieve permanent expression of the CRISPR-Cas9 system by integrating into the host genome. That is, gene transfection, this property has played a significant role in the treatment of diseases such as sickle cell anemia (SCD), β-thalassemia, and in CAR-T therapy. However, it is precisely this property that can lead to long-term genetic toxicity in host cells. For instance, the integration of LV near oncogenes may lead to their activation, thereby causing tumorigenesis. The development of integration-defective lentiviruses using plasmids expressing mutant integrases may enhance the safety of LV transduction. However, it is still difficult to avoid the occurrence of integration to varying degrees [13,14]. In contrast, LVs have a larger vector capacity than AAV vectors, approximately 7kb, compared to less than 8kb. As a result, they can carry more components of the Cas9 nuclease gene and sgRNA.

## 2.2. Nanoparticles

Nanoparticles (NPs) are defined as materials with a size ranging from 1 to 100 nanometers. Their unique biological properties are mainly determined by the changes in physical and chemical attributes brought about by the significantly increased specific surface area. Nanoparticles can be obtained through multiple synthetic routes, including chemical synthesis and spontaneous molecular self-assembly. Based on these characteristics, as well as the improvement of drug solubility, stability and targeting ability, nanoparticles have been widely applied in the biomedical field, especially in drug delivery systems.

### 2.2.1. Lipid nanoparticles (LNP)

Lipid nanoparticles (LNPs) are essentially formed by enveloping negatively charged nucleic acids, such as sgRNA, Cas9 mRNA or RNP in the CRISPR-Cas9 system, with positively charged lipids,

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resulting in particles with a diameter of 50-100 nm. These particles enter cells through endocytosis and release their internal cargo [13,15]. Its characteristics include simple preparation, convenient surface modification, and low likelihood of triggering immune responses, etc. Lipid nanoparticles have an external lipid that can be composed of multiple components. Among them, there are four key components. Ionizable cationic lipids, which protonate in acidic environments and mediate endosomal escape. Zwitterionic phospholipids, which serve as the structural framework and form the basis of the bilayer. Cholesterol, which enhances membrane stability by filling the lipid bilayer. PEGylated lipids, which improve the colloidal stability of nanoparticles through steric hindrance effects. Lipid nanoparticles mainly include: SLN, NLC, liposomes and noise bodies. Lipid nanoparticles mainly include: SLN, NLC, liposomes and noise bodies.Because Cas9 RNP complexes are large and negatively charged, they poorly penetrate the mammalian cell membrane, which is negatively charged [16]. A core solution for the intracellular delivery of negatively charged Cas9 RNP is to employ cationic liposomes. The principle utilizes the attraction between positive and negative charges, that is, it enables cationic lipids to spontaneously combine with negatively charged nucleic acids through electrostatic interaction. While enhancing the drug loading efficiency, the final formed LNP as a whole can accelerate the endocytosis of cells through local electrostatic interactions and the formation of a protein corona, enabling the Cas9 RNP to effectively enter the interior of cells.

Lipid nanoparticles have natural liver-targeting properties and will accumulate in large quantities in the liver after intravenous injection, so they are often used to treat liver-related diseases, such as transthyretin amyloidosis ATTR and hypercholesterolemia [17]. At the same time, local delivery methods can be used, such as injecting into the tumor for gene editing or treating neuromuscular diseases like Duchenne muscular dystrophy [18]. The lipid components that modify the external liposomes can be used to target other organs, such as treating neurodegenerative diseases by targeting the brain.

### 2.2.2. Polymer nanoparticles (PNPs)

In analogy to lipid nanoparticles, polymer nanoparticles also electrostatically bind the components of the CRISPR-Cas9 system through cationic polymers and enter cells via adsorption on the cell membrane or receptor-mediated endocytosis. Polymer nanoparticles mainly include: micelles, dendrimers, vesicles and hydrogels.

The core mechanism of cationic polymers as delivery vectors for the CRISPR/Cas9 system lies in the formation of stable nanocomplexes with nucleic acid components through electrostatic adsorption.They can self-assemble into functionalizable nanocomplexes and integrate multiple functions through chemical modification: for instance, attaching cell-penetrating peptides (CPPs) or specific targeting ligands to enhance cellular uptake and tissue targeting; or incorporating polyethyleneimine to buffer the endosomal pH and promote membrane rupture, facilitating efficient endosomal escape [19]. Compared with the relatively simple chemical structure of cationic lipids, the monomer structure, molecular weight and configuration of polymers can all be adjusted, which brings strong functional potential. In addition to serving as independent carriers, polymers can also be integrated into cationic LNP as functional components, taking advantage of their film-forming property or spatial stability to synergistically enhance the targeted delivery efficiency of CRISPR/Cas9 complexes in vivo [20].

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### 2.2.3. Inorganic nanoparticles (INPs)

In non-viral delivery systems, inorganic nanomaterials offer new delivery options beyond lipid and polymer carriers due to their stable structure, loading capacity and chemical inertness. Among the most promising ones, there are gold nanoparticles (AuNPs), which focus on biosafety, mesoporous silica nanoparticles (MSNs), which have high loading capacity, and biomimetic zeolitic imidazolate frameworks (ZIFs), which are able to deliver to individual cells. Gold nanoparticles (AuNPs) take pride in their chemical inertness and hence they can easily escape immune responses making them highly effective and low-toxicity gene editing conduction instruments [21].

Additionally, their surfaces can be easily modified with various ligands through gold-sulfur bonds, providing convenience for targeted delivery. Mesoporous silica nanoparticles (MSNs) have the advantage of high specific surface area and pore structure, which can be used to load a large amount of CRISPR/Cas9 components [22]. More importantly, the abundant silanol groups on their surface facilitate extensive chemical modification, which can improve their biodistribution and cellular uptake efficiency, thereby optimizing the delivery performance of CRISPR/Cas9. As a typical biomimetic MOF, zeolitic imidazolate framework (ZIF-8) has demonstrated unique cell specificity in delivering CRISPR/Cas9. By coating ZIF-8 nanoparticles with cancer cell membranes, homologous targeting is utilized to enable the CRISPR/Cas9-carrying vectors to be recognized and endocytosed by specific cancer cells, achieving precise gene editing therapy [23].

## 2.3. Physical delivery

Compared with non-viral methods in chemistry and biology, physical delivery methods (such as microinjection, electroporation and hydrodynamic injection) are more straightforward in operation. They penetrate the membrane of the cell (including the nuclear one) with physical force and deep into the cell interior, directing CRISPR/Cas9 to the location of interest, thereby bypassing the complicated transport mechanism [13,24]. Whereas these methods have proven their excellent efficiency in in vitro experiments and in laboratories, its application in the treatment of animals is not yet well developed. This is because they have technical shortcomings such as low scalability, which complicates their ability to support treating the systemic treatment; shallow depth of tissue penetration and high reliance on the competence of the operating personnel and special equipments.

### 2.3.1. Microinjection

Microinjection is a key technique for the precise delivery of CRISPR/Cas9 in the laboratory [16]. The operation is a highly controlled process: in a precise microscopic operating system, the target cells are first fixed, and then a glass microneedle with a tip diameter at the micrometer level is manipulated to pierce the cell membrane and release the contents directly into the cytoplasm. The advantage of this technology lies in its directness and quantitativeness. It physically crosses the cell membrane, ensuring the efficient in-situ delivery of the editing tools. It is consequently employed in single-cell gene editing. Generally, it is applied to reproductive cell editing or to correct the genes of embryos in vitro fertilization.

### 2.3.2. Electroporation

The core of electroporation technology lies in applying short and controllable high-voltage pulses to temporarily restructure the phospholipid bilayer of the cell membrane, creating hydrophilic transient pores. As a result, large molecules that were originally unable to penetrate the cell membrane can

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now pass through it [25]. Due to its high efficiency and universality, in vitro electroporation has become one of the preferred strategies for introducing CRISPR/Cas9 into relevant cells, such as CAR-T cells or hematopoietic stem cells, and holds great clinical potential. For instance, cell editing can be carried out in vitro, such as editing T-cells or hematopoietic stem cells, to treat sickle cell anemia. Or it can be delivered locally to the skin or muscle tissue within the body.

### 2.4. Emerging efficient delivery methods

After development, on the basis of the original viral vector delivery and nanoparticle polymer delivery, many emerging delivery methods have been developed. This includes technologies such as virus-like particles (VLPs), exosomes, and engineered extracellular vesicles (EVs).

#### 2.4.1. Extracellular vesicle delivery (EVs)

As endogenous nanocarriers, extracellular vesicles with a diameter of 30 to 150 nm originally mediate intercellular communication by transporting biological macromolecules in the body, and thus have characteristics such as non-immunogenicity, high stability and safety. This delivery vectors can load RNP to achieve efficient gene editing. By expressing molecules for specific recognition on the membrane surface, it can endow EVs with targeted delivery ability and significantly improve the precision of gene editing [26].

Exosomes are a specific subclass of extracellular vesicles, with a diameter ranging from 0 to 100 nm. It can transfer various signal molecules, including mRNA and microRNA, etc. Due to its small size, it can pass through some biological barriers, such as the blood-brain barrier. However, precisely because of its small size, it is unable to fully load the genes required for expressing Cas9 and can only carry some small nucleic acids or low-molecular-weight drugs. An alternative strategy for encapsulating the CRISPR-Cas9 expression vector involves incubating exosomes with liposomes to form hybrid exosomes [27].

#### 2.4.2. Virus-like particle-mediated (VLP)

Different from the viral vector delivery, virus-like particles (VLPs) possess the components that normal viral vectors have, such as envelopes and capsids, but they lack the viral genome [28]. The capsid can serve as a tool for delivering viral genes and also play a role in delivering the CRISPR/Cas9 system, thereby achieving more efficient gene editing. At present, the viral (LV) capsid proteins that are used more frequently are mainly those of lentiviruses.

## 3. Neurological diseases

Neurological disorders refer to a category of diseases caused by structural, biochemical or functional abnormalities of the nervous system. The types include neurodegenerative diseases, neurodevelopmental disorders, episodic brain function disorders, neuromuscular diseases, cerebrovascular diseases, neuroinfectious and inflammatory diseases, traumatic and toxic neurological diseases, also genetic metabolic diseases of the nervous system. In this part of the review, a category of diseases with complex pathogenesis and suitable for treatment with gene editing techniques will be selected [2]. The blood-brain barrier (BBB) is one of the obstacles in the treatment of central nervous system diseases. It is mainly composed of brain microvascular endothelial cells (BMECs), and works in concert with pericytes, astrocyte end feet, and the

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basement membrane to form the neurovascular unit, thereby achieving the effect of controlling the entry and exit of substances into and out of the brain tissue.

The pathological hallmark of neurodegenerative diseases is the irreversible death of neurons and the abnormal deposition of proteins. Here, Alzheimer's disease, a relatively typical example, is taken as an illustration. In Alzheimer's disease, autopsy results show the formation and deposition of amyloid beta (Aβ) and phosphorylated tau in the brain, which leads to extracellular amyloid plaques and neurofibrillary tangles [2].The pathogenesis of Alzheimer's disease is complex. The vast majority of patients (90%) have sporadic cases, which are usually related to multiple factors such as the external environment and diseases [29]. In familial Alzheimer's disease (FAD), three genes are the main factors causing AD: amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) [30]. The synthesis of β-amyloid protein is a proteolytic cascade protein, and it starts with the cleavage of amyloid precursor protein by β-secretase to produce a C99 fragment tied to the membrane. Later, the erythrometric fragment is cut into two parts by the erythrometric γ-secretase complex, producing Aβ1-40 peptides as the major product and fewer but more toxic Aβ1-42 peptides. The two other hydrophobic amino acids that are found at the C-terminus of Aβ1-42 greatly augment the hydrophobicity of this protein such that it is more apt to misfold and self-assemble, thus leading to the establishment of neuroinflammatory amyloid plaques.

To address this, the C-terminal fragment of APP protein can be removed through CRISPR, blocking the cleavage by β-secretase, reducing the generation of Aβ, and simultaneously increasing the neuroprotective fragment sAPPα. Simultaneously, the generation of BACE1, an enzyme involved in the production of Aβ, can also be reduced through CRISPR-Cas9 technology. Additionally, the expression of brain-derived neurotrophic factor (BDNF), a type of neurotrophic factor that enable to enhance neuronal protection and repair synapses, can be enhanced through CRISPR-Cas9 technology.

In clinical selection, AAV vectors remain the preferred vectors for treating neurodegenerative diseases. AAV9 and AAV-PHP.B can cross the BBB and infect neurons/glial cells after intravenous injection, and their effective duration is relatively long. After intravenous injection, exosomes modified with Lamp2b-RVG peptide can accumulate in the brain at a rate of up to 30%. Moreover, compared with AAV, it has lower immunogenicity and can target microglia for modification against CD47. Neurotropic LV vectors are also a good choice because of their ability to integrate into the host cell genome, which can achieve long-term expression [11].

## 4. Conclusion

To date, the CRISPR-Cas9 system has been studied in depth and various delivery methods for different applications have been developed, including viral vector delivery, physical delivery, and nanoparticle delivery, etc. However, in most cases, it is difficult to fully cover all aspects of safety, effectiveness and cost. At such times, adjustments are made through methods such as chemical modification. In clinical practice, for different diseases and based on the different locations and targets of the diseases, in addition to choosing a single delivery method, using multiple methods for co-delivery can become a new direction for consideration. Meanwhile, in addressing the issue of eliminating the toxicity of viral vector delivery, virus-like particle-mediated delivery offers a promising solution with a bright future.

Owing to their complex pathogenesis, neurodegenerative diseases present a major challenge for therapeutic delivery. It is crucial to use delivery technologies that are low in immunogenicity, highly neuro-targeted, and capable of penetrating the blood-brain barrier. New delivery methods developed on the basis of the original viral vector delivery, such as engineered exosomes and modified lipid

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nanoparticles, can all effectively solve this problem. It is necessary to edit multiple genes and multiple cells. The CRISPR-Cas9 system holds great promise in this regard.

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