Review Article

CRISPR/CAS Platforms and their Delivery Systems: A Comprehensive Overview

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ABSTRACT

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a revolutionary gene-editing tool derived from the adaptive immune system of bacteria and archaea. The CRISPR/Cas system allows for precise, efficient, and cost-effective genome modification across various organisms. Since its development, CRISPR technology has rapidly advanced, expanding to include enhanced systems like Cas12, Cas13, Cas14, base editors, and prime editors. These systems provide new possibilities in plant biotechnology, biomedical research, diagnostics, and therapeutic gene editing. CRISPR-based delivery methods—both viral and non-viral—are critical to the success of genome engineering. Emerging nanocarriers and physical delivery methods offer promising strategies to overcome biological barriers and improve targeted delivery. With widespread applications in agriculture, infectious disease treatment, and cancer therapy, CRISPR represents a transformative platform in modern science.

Keywords: CRISPR/Cas9, Genome editing, Viral vectors, Non-viral delivery, Targeted gene delivery.

INTRODUCTION

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is an adaptive immune mechanism in bacteria and archaea that protects against invading genetic elements such as plasmids and bacteriophages. This system functions as a form of genetic memory, where fragments of foreign DNA (spacers) are integrated between repeated sequences within the CRISPR locus. Upon re-exposure to the same or similar genetic material, the CRISPR array is transcribed into CRISPR RNA (crRNA), which guides the Cas9 endonuclease to the matching target sequence. A single-guide RNA (sgRNA), combining crRNA and trans-activating crRNA (tracrRNA), directs Cas9 to specific DNA sites adjacent to a protospacer adjacent motif (PAM), typically the sequence "NGG." Cas9 then introduces a precise double-strand break at the target site. The specificity and efficiency of this system have made CRISPR-Cas9 a transformative tool in genome editing, molecular diagnostics, and therapeutic applications [1].

The CRISPR/Cas system was first observed in 1987, although its functional significance remained unclear at the time. Subsequent studies revealed its widespread presence—occurring in approximately 90% of archaeal species and nearly 40% of bacterial genomes. By 2005, it was recognized as a crucial element of the prokaryotic adaptive immune system, primarily defending against bacteriophage infections. A landmark advancement occurred in 2012 when scientists successfully adapted the CRISPR/Cas mechanism for targeted genome editing in eukaryotic cells[2].

Compared to earlier gene-editing tools, CRISPR/Cas offers distinct advantages, including lower cost, greater precision, and higher efficiency, establishing it as a versatile platform in modern genetic engineering and biomedical research [3].

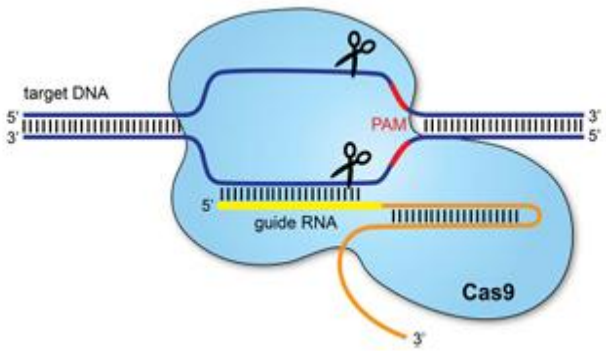

Figure 1: Mechanism Underlying CRISPR-Cas9–Mediated Genome Editing [4]

Genome editing (GE) refers to a set of molecular techniques used to make precise, targeted modifications to an organism's DNA sequence [5]. These modifications may include insertions, deletions (indels), or single-nucleotide substitutions at specific genomic loci [6]. Several genome editing technologies have emerged over the years, each designed to make precise changes in DNA. These include tools such as

- Zinc Finger Nucleases (ZFNs): These are synthetic proteins designed to bind specific DNA sequences and induce double-stranded breaks (DSBs) [7]. First utilized in 2002, ZFNs were initially applied across various species. However, they are limited by low target specificity, frequent off-target activity, and the technical complexity of constructing expression vectors [8].
- Transcription Activator-Like Effector Nucleases (TALENs): Introduced in 2011, TALENs also generate DSBs at specific genomic sites to initiate DNA repair and subsequent gene modification [9]. Despite improved targeting flexibility compared to ZFNs, TALENs suffer

from similar drawbacks, such as labor-intensive vector assembly and delivery challenges [10].

Due to the inherent limitations of ZFNs and TALENs, the focus of genome editing has shifted since 2013 to the CRISPR/Cas9 system. This RNA-guided approach employs the Cas9 endonuclease, directed by a single-guide RNA (sgRNA), to cleave DNA at desired loci through complementary base pairing [11].

Compared to earlier methods, CRISPR/Cas9 is more cost-effective, user-friendly, and adaptable, making it applicable across diverse biological systems including plants, animals, and humans [12].

Recent advances in CRISPR technology have led to the development of enhanced systems such as [13]:

- Base Editing (BE)
- xCas9
- Cas12a (Cpf1)
- Cas13

These variants offer improved specificity, editing efficiency, and novel functionalities, particularly useful in crop improvement and plant biotechnology research [14].

TYPES OF CRISPR-CAS SYSTEMS

The CRISPR/Cas systems are generally categorized into two primary classes, depending on the structure and components of their effector complexes:

- Class 1 systems (Types I, III, and IV): These involve a multi-protein effector complex that collaborates to cleave nucleic acids [15-16].
- Class 2 systems (Types II, V, and VI): These utilize a single, multifunctional Cas protein, such as Cas9, Cas12, Cas13, or Cas14, to carry out the cleavage [17].

Due to the complexity of expressing multiple subunits, Class 1 systems are not commonly employed in eukaryotic genome engineering. In contrast, Class 2 systems—which are easier to manipulate and deliver—are extensively used in gene therapy, biotechnology, and molecular biology.

Among these, Cas9, Cas12, Cas13, and Cas14 have emerged as vital genome-editing tools, especially in applications involving therapeutic gene modification and functional genomics [18]. The CRISPR/Cas9 system has emerged as one of the most widely utilized genome editing tools due to its precision, versatility, and efficiency in modifying genes across diverse organisms [19].

1. Mechanism of Action**:** Cas9 acts as an RNA-guided nuclease by assembling into a ribonucleoprotein (RNP) complex with CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA), enabling precise DNA targeting. This complex targets and introduces double-stranded breaks (DSBs) at specific DNA loci [20].
2. PAM Sequence Requirement: The commonly used *Streptococcus pyogenes* Cas9 (SpCas9) recognizes a 5′-NGG-3′ protospacer adjacent motif (PAM), while *Staphylococcus aureus* Cas9 (SaCas9) recognizes a broader 5′-NNGRRT motif, enabling more flexible targeting [21].
3. DNA Repair Pathways: Following DSBs, cellular repair occurs via either non-homologous end joining (NHEJ)—which often results in insertions or deletions—or homology-directed repair (HDR) for precise gene editing[22].
4. Limitations: Challenges with Cas9 include off-target cleavage, dependence on specific PAM sequences, and difficulties in delivering the system to target tissues.
5. Enhanced Variants: To overcome these limitations, advanced modifications such as dead-Cas9 (dCas9), base editors, prime editors, and engineered Cas9 variants have been developed to increase precision and expand functionality.
6. Ongoing Relevance: Despite newer alternatives, CRISPR/Cas9 continues to serve as a foundational tool in gene editing, disease modeling, and therapeutic research [23].

CRISPR/Cas12 System:

Introduced in 2015, the Cas12 family—formerly referred to as Cpf1—provides an alternative approach to Cas9-based genome editing.Cas12a targets DNA sequences flanked by a 5′-TTTV-3′ PAM and generates staggered (sticky) ends, which improve the efficiency of HDR-based insertions [24].

- Upon activation, Cas12a exhibits collateral cleavage activity against single-stranded DNA (ssDNA), which has been exploited in viral diagnostics.
- Cas12a remains active post-cleavage due to the distant location of the PAM from the cleavage site, improving targeting accuracy.
- Cas12b, a smaller and more precise variant, has shown promise in genome editing, though early forms were limited by high-temperature requirements. Engineered versions now function effectively at physiological temperatures (37°C) [25-26].

CRISPR/Cas13 System: RNA-Targeting Technology

Discovered in 2016, CRISPR/Cas13 represents a departure from DNA-targeting systems by specifically targeting RNA molecules [27].

- Cas13 requires only a crRNA for guidance and, upon recognition of target RNA, initiates collateral degradation of nearby non-target RNAs through indiscriminate cleavage activity [28].
- The Cas13 family includes Cas13a, Cas13b, Cas13c, and Cas13d (CasRx).

- Although the detailed mechanism in eukaryotic cells is still being investigated, Cas13 shows significant potential in RNA diagnostics and therapies, including applications targeting mRNA, microRNA, and long non-coding RNAs (lncRNAs) [29].

CRISPR/Cas14: A Compact System for ssDNA Editing

Discovered in 2018 from archaea, Cas14 is a minimalistic CRISPR nuclease, roughly one-third the size of Cas9, and harbors a RuvC-like domain for endonuclease activity [30].

- Cas14 selectively cleaves single-stranded DNA (ssDNA) without requiring a strict PAM sequence, offering improved targeting specificity over Cas12a.
- Its compact structure and high precision make Cas14 a promising tool in molecular diagnostics, particularly for detecting genetic mutations, pathogens, and tumor biomarkers [31]. Gene editing encompasses a range of molecular techniques that enable targeted alterations in the DNA sequence at specific genomic sites. Traditional approaches often utilize programmable nucleases to introduce double-strand breaks (DSBs), which are subsequently repaired by cellular mechanisms such as non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), or homology-directed repair (HDR) [32].

Among the most transformative technologies is the CRISPR-Cas system, originally identified as part of the adaptive immune defense in prokaryotic organisms. This system provides sequence-specific targeting of invading genetic elements such as plasmids or viruses [33]. The widely used Type II CRISPR-Cas9 system employs the Cas9 endonuclease and a single-guide RNA (sgRNA) to locate and bind DNA sequences adjacent to a protospacer adjacent motif (PAM), typically 5′-NGG. Cas9 then cleaves the DNA near the target, enabling precise genome modification [34].

Due to its simplicity, cost-efficiency, and high precision, the CRISPR-Cas system has rapidly become the tool of choice in gene editing. Numerous Cas9 orthologs and engineered variants have been developed to increase targeting range and reduce off-target effects. Furthermore, next-generation tools such as base editors (BEs) and prime editors (PEs) allow for specific nucleotide changes without the need for DSBs, greatly enhancing the safety and versatility of genome engineering applications [35].

Mechanism of CRISPR in Drug Delivery: A Prokaryotic Defense Model

The CRISPR-Cas system originates as an adaptive immune defense in prokaryotes, offering protection against invading genetic elements such as viruses (bacteriophages) and plasmids [36]. This process serves as the molecular basis for leveraging CRISPR in modern gene therapy and pharmacological delivery platforms

1. Adaptive Immunity in Prokaryotes

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) loci serve as genomic repositories of prior infections. These sequences capture short fragments of invading DNA, referred to as spacers, and integrate them into the host CRISPR array, creating a genetic "memory" of past exposures [37].

2. RNA Processing and Target Recognition

Once a pathogen is recognized, the CRISPR array is transcribed into a long precursor RNA (pre-crRNA), which is then cleaved into smaller units known as crRNAs. These crRNAs contain sequence-specific information that guides Cas proteins to complementary regions in foreign DNA or RNA [38].

3. Target Cleavage and Elimination

In *E. coli*, the crRNA-bound Cas protein complex—such as the CASCADE complex—identifies complementary sequences within invading genetic elements. Upon finding a match adjacent to a PAM (Protospacer Adjacent Motif), the complex binds and cleaves the foreign DNA, effectively neutralizing the threat [39].

Stages of the CRISPR-Cas Immune Response:

The CRISPR-Cas system operates through a well-defined sequence of stages:

a. Adaptation (Spacer Acquisition):

In the initial stage, fragments of foreign DNA (protospacers) are captured and integrated at the leader end of the CRISPR locus. This integration process, facilitated primarily by Cas1 and Cas2 proteins, requires a PAM sequence to ensure accurate target recognition during future encounters [40].

b. Expression:

The entire CRISPR array is transcribed into pre-crRNA, which is then processed by Cas endonucleases into individual crRNAs. These act as sequence-specific guides, enabling the system to detect previously encountered genetic invaders[41].

c. Interference:

In this final phase, crRNAs associate with Cas effector proteins to form surveillance complexes. These complexes identify and bind to foreign DNA that contains a matching sequence and a valid PAM motif. If these conditions are met, the DNA is cleaved and degraded. Mutations in the target sequence or PAM can prevent cleavage, potentially allowing evasion by the pathogen [42-43].

This mechanism has been repurposed in therapeutic applications, including gene correction, targeted gene silencing, and controlled drug delivery, where the programmable nature of the CRISPR-Cas system allows for precise manipulation of genetic material in target cells.

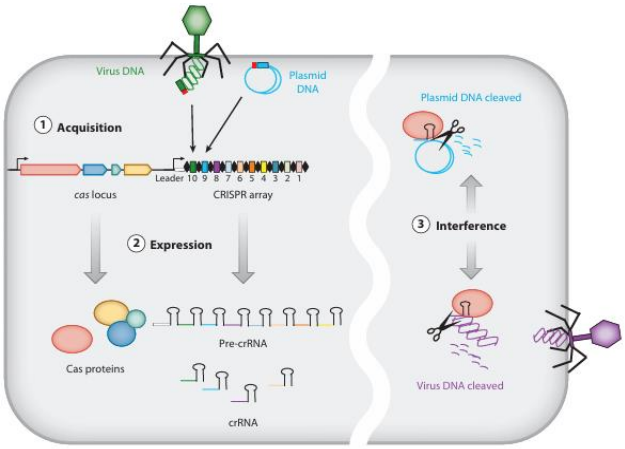

Figure 2: Schematic representation of the CRISPR adaptive immune system in prokaryotes [44].

Key Features of the CRISPR-Cas System:

1. Heritable Immunity:

One of the most remarkable aspects of the CRISPR-Cas system is its ability to confer heritable adaptive immunity. Once foreign DNA fragments (spacers) are integrated into the host CRISPR array, this immunological memory is passed on to progeny cells, allowing them to recognize and neutralize similar threats. This form of inheritance mirrors a Lamarckian mechanism, where acquired traits (spacer sequences) are transmitted across generations [45].

2. Dynamic and Adaptive Defense:

The CRISPR-Cas system is highly dynamic, capable of acquiring new spacers in response to novel infections and losing outdated ones over time. This adaptability ensures effective defense against rapidly mutating viruses and other mobile genetic elements [46].

3. Structural Components of the System

- **Spacer Incorporation:** During the adaptation phase, short DNA fragments from invading elements—called spacers—are inserted into the host's CRISPR locus. These spacers encode sequence-specific immunity by serving as molecular records of prior invasions [47].
- **CRISPR Locus:** The CRISPR locus consists of alternating repeat and spacer sequences, forming the genetic backbone of the immune memory [48].
- **Cas Genes:** Located near the CRISPR array, CRISPR-associated (cas) genes code for proteins essential to spacer acquisition, crRNA maturation, and interference with target sequences. These include critical proteins like Cas1, Cas2, Cas9, Cas12, and others depending on the system type [49].

4. Three Essential Functional Stages:

The CRISPR-Cas system operates through three distinct but interdependent phases:

- **Adaptation:**
  Insertion of short foreign DNA segments (protospacers) into the CRISPR locus, establishing molecular memory of prior infections.
- **Expression:**
  Transcription of the CRISPR array into precursor crRNA (pre-crRNA), followed by its cleavage into functional guide crRNAs.
- **Interference:**
  Targeted binding and cleavage of complementary invading DNA or RNA sequences by the crRNA-Cas effector complex [50].

All three stages are essential for the system to function as an effective immune mechanism in prokaryotes and serve as the foundation for its use in gene editing and therapeutic applications.

The CRISPR/Cas9 genome editing tool originates from the bacterial CRISPR system, which functions as part of the bacterial immune response. CRISPR/Cas9 technology has gained significant popularity due to its high specificity and efficiency in genome editing. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) locus and its associated proteins are primarily found in certain bacteria and are involved in providing immunity against phage infections [51].

Types of CRISPR Delivery Systems

1. Viral Delivery Systems

A variety of viral vectors have been employed to transport CRISPR-Cas9 components into mammalian cells due to their efficiency and ability to target specific tissues.

- Owing to their strong potential in gene and cell therapy, vectors such as **adenoviruses**, **adeno-associated viruses (AAVs)**, **lentiviruses**, and **baculoviruses** are frequently used for delivering CRISPR-Cas components with high accuracy and efficiency [52].
- Viral vectors are naturally adept at infecting host cells, making them attractive candidates for gene delivery. An ideal viral delivery system should (1) support the cargo size of therapeutic genes, (2) be straightforward to produce and package, (3) be replication-deficient to ensure biosafety, and (4) provide effective transduction *in vivo* [53].

a) Adeno-Associated Virus (AAV)

AAVs have shown considerable therapeutic potential and a favorable safety record in both animal studies and human clinical trials. Because of their non-pathogenic nature, high transduction efficiency, and compatibility with genetic engineering, AAVs are widely considered among the most effective viral vectors for delivering CRISPR elements [54]. Specialized AAV-based vectors have been developed to deliver Cas9 and guide RNAs—either individually or together—into hard-to-transfect cells. Packaging these components into engineered AAV capsids enables precise and efficient genome editing [55].

b) Adenoviruses

Adenoviruses (AdVs) are non-enveloped, double-stranded DNA viruses with an icosahedral structure. Capable of infecting both proliferating and non-proliferating cells, their genome remains episomal post-delivery—avoiding integration into the host genome and reducing off-target mutation risks [56].

With a genome size of 34–43 kb and no strict cargo size limits, AdVs can accommodate larger CRISPR constructs than most other viral vectors. However, due to their common presence in humans, they may trigger strong immune responses, potentially causing inflammation and rapid clearance from the body [57].

c) Lentiviruses

Lentiviral vectors are frequently used in both *in vitro* and *ex vivo* applications due to their efficiency in transducing a wide range of cell types, including dividing and non-dividing cells. They also offer a cloning capacity of up to ~8 kb and require less complex production compared to AAVs [58]. Lentivirus-based CRISPR/Cas9 systems have shown promise in the treatment of diseases such as **chronic hepatitis B** and **HIV**, where the system successfully removed integrated HIV DNA from host T cells [59]. Additionally, lentiviral delivery has been demonstrated to lower intraocular pressure in mouse models of glaucoma, highlighting its therapeutic potential[60].

d) Baculoviruses

Baculoviruses are large, double-stranded DNA viruses specific to arthropods and belong to the *Baculoviridae* family. Their structural capacity and ability to carry large gene payloads make them attractive for CRISPR delivery applications in specific research contexts [61].

2. Non-Viral Delivery Systems

Delivering CRISPR-Cas9 agents directly into the bloodstream presents multiple challenges. These include potential off-target gene editing in healthy tissues, which could lead to adverse effects. Moreover, the therapeutic agents often suffer from rapid degradation and limited stability within the biological environment [62]. Nucleic acids such as DNA and RNA are especially susceptible to enzymatic breakdown and have difficulty crossing cell membranes due to their hydrophilic, negatively charged nature.

To overcome these obstacles, nano carrier-based **strategies** have been widely investigated. These nano scale delivery vehicles offer enhanced protection against degradation, improved cellular uptake, and the ability to direct CRISPR components to specific tissues or cells. Selecting the right delivery platform is crucial for achieving high precision and safety in genome editing therapies. Recent advances in nanotechnology have enabled the creation of multifunctional carriers capable of bypassing biological barriers and improving delivery performance [63].

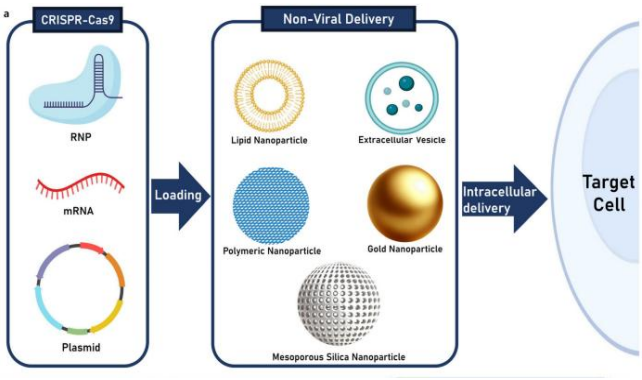

Figure 3: *Summary of non-viral strategies for CRISPR-Cas9 delivery and genome editing.* (a) A range of non-viral platforms—including lipid-based carriers, polymeric nanoparticles, inorganic materials, and physical methods—are employed to load and transport CRISPR-Cas9 components into target cells [64].

Lipid-Based Nanoparticles (LNPs)

Lipids, including both natural and synthetic varieties such as fatty acids and phospholipids, are widely used in drug delivery. Among these, **amphiphilic phospholipids** are of particular importance as they form the basis of cell membranes and liposomal structures. Liposomes—spherical vesicles with bilayer membranes—can encapsulate both hydrophilic and hydrophobic molecules, gaining entry into cells through endocytosis.

Cationic liposomes, which carry a positive charge, are capable of binding to negatively charged DNA or RNA via electrostatic interaction. This forms stable complexes that shield the nucleic acids from enzymatic degradation and enhance their uptake by target cells. These features make lipid-based vectors, especially liposomes, a promising approach for CRISPR-Cas9 delivery [65].

In this strategy, **positively charged liposomes interact with negatively charged nucleic acids**, forming lipid nanoparticles (LNPs) that facilitate cellular entry and avoid immune degradation [66].

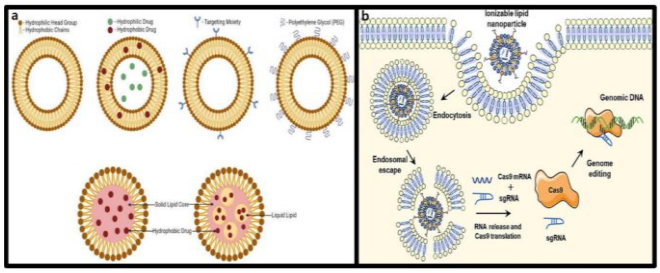

Figure 4: *Design features of lipid nanoparticles (LNPs) and their role in CRISPR-Cas9 delivery.* (a) Various LNP architectures and surface modifications tailored for targeted gene transfer [67]. (b) Intracellular transport and activation pathway of CRISPR-Cas9 using LNP-mediated delivery [68].

Polymeric Nanoparticles:

Polymer-based nanomaterials have shown considerable promise in gene delivery applications due to their versatile properties and capability to transport a variety of nucleic acids efficiently [63]. Polyethyleneimine (PEI) is one of the most commonly used polymers for nanoparticle formulation, owing to its high gene transfection efficiency[69].

Polymeric delivery systems are emerging as a safer alternative to viral vectors, offering high cargo capacity and a favorable biocompatibility profile [70]. Unlike cationic lipids, cationic polymers exhibit greater structural diversity and functional tunability, enabling customized nanoparticle designs for specific applications. lipid-based systems, polymeric nanoparticles typically enter cells via endocytosis and provide protection to the nucleic acid cargo from nucleases and immune recognition, thus enhancing intracellular stability and delivery efficiency [71].

Cationic Polymers in Cas9 Plasmid Delivery:

Cationic polymers have been effectively utilized for delivering Cas9 plasmids, which can be engineered to include not only the Cas9 gene but also the single-guide RNA (sgRNA) and homology-directed repair (HDR) templates, all within a single construct. Compared to mRNA or protein-based systems, plasmid DNA offers enhanced stability during storage and handling.

However, plasmid-based delivery has some notable limitations:

1. Delayed therapeutic action, as plasmids must first enter the nucleus and undergo transcription and translation before Cas9 becomes functional.
2. Prolonged Cas9 expression from persistent plasmids may increase the likelihood of off-target genome editing.
3. The nuclear import requirement of plasmid DNA can hinder editing efficiency, especially in non-dividing or slowly dividing cells.

Despite these drawbacks, cationic polymers remain attractive due to their ability to condense and protect large plasmid constructs, facilitating cellular uptake and endosomal escape [72].

Gold Nanoparticles (AuNPs) in CRISPR Delivery:

The unique properties of metal-based nanoparticles, such as gold nanoparticles (AuNPs), including enhanced stability, efficient cellular uptake, and ease of functionalization, make them ideal candidates for CRISPR delivery agents [73-74]. AuNPs are highly valued for their biocompatibility, optical tunability, and surface modifiability, which allow for ligand functionalization to achieve targeted delivery [75]. These nanoparticles have found diverse applications in biomedical research, including imaging, drug delivery, and gene editing, and have been utilized in *in vitro, ex vivo*, *and in vivo* settings [76]. The CRISPR-Gold system, for instance, utilizes AuNPs conjugated with Cas9 ribonucleoprotein and donor DNA to enhance genome editing efficiency in animal models [77]. Furthermore, their ability to be functionalized with targeting ligands increases delivery precision, and their compact size and prolonged circulation improve therapeutic impact [78].

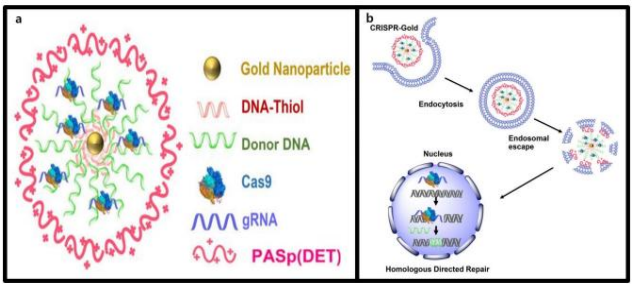

Figure 5: *Optimized CRISPR-Cas9 delivery using the structural features of gold nanoparticles (AuNPs).* (a) AuNPs are functionalized with thiolated oligonucleotides (DNA-Thiol), hybridized with single-stranded donor DNA (ssODNs), and assembled with Cas9 ribonucleoproteins (RNPs) and the endosome-disrupting polymer PAsp(DET), where "DET" denotes diethylenetriamine. (b) The CRISPR-Gold complex enters cells through endocytosis, promotes endosomal escape, and releases Cas9 RNPs and donor templates into the cytosol to enable genome editing [79].

Physical Delivery System:

Microinjection:

Microinjection is widely regarded as the "gold standard" for delivering CRISPR-Cas9 components into individual cells due to its high efficiency, often nearing 100%. This technique involves the direct injection of CRISPR materials—such as plasmid DNA encoding Cas9 and sgRNA, Cas9 mRNA with sgRNA, or pre-assembled Cas9 ribonucleoprotein complexes—into the cytoplasm or nucleus of target cells using a fine glass micropipette (typically 0.5–5.0 µm in diameter) under a microscope. The method enables precise intracellular delivery while bypassing cellular uptake barriers, thereby enhancing genome editing accuracy and minimizing off-target effects[80].

This method was initially demonstrated by the medical researcher Barber, who showed that microinjection allows the direct delivery of substantial amounts of genetic material into cells in a controlled manner. As a gene delivery strategy, microinjection is recognized for its non-toxic nature, compatibility with living cells, reproducibility, and affordability.It enables precise introduction of CRISPR-Cas9 components, offering high efficiency with minimal cellular toxicity. Despite these advantages, the method requires skilled handling, involves the manipulation of individual cells, and is labor-intensive. Additionally, to prevent disruption of the cell membrane and intracellular structures, only a small volume of genetic material can be injected. Due to these constraints, microinjection is mainly suitable for larger cells such as oocytes and certain oncocytes [81].

Electroporation:

Electroporation is a commonly used physical technique for delivering nucleic acids and proteins into mammalian cells. This technique transiently increases the permeability of the cell membrane through the application of electrical pulses, thereby facilitating the entry of biomolecules into the cytoplasm or nucleus. It is applicable to various forms of CRISPR-Cas9 systems, including plasmid-based systems, Cas9 mRNA with sgRNA, and ribonucleoprotein (RNP) complexes. However, the method has notable limitations, such as a low plasmid DNA integration efficiency—reported to be around 0.01% in target cells—and a high rate of cell death due to membrane disruption caused by electrical pulses [82].

Electroporation is a widely used technique that temporarily increases the permeability of the cell membrane by applying an electric field, forming nanometer-sized pores. These pores allow negatively charged DNA or other genetic materials, which are typically non-permeable, to enter the cytoplasm or nucleus, enabling effective gene delivery [83]. The strength and duration of electric pulses vary depending on the target cell type. For instance, in skeletal muscles, a high-voltage pulse is initially applied to form membrane pores, followed by low-intensity pulses that guide the genetic material through these pores via an electrophoretic effect [84]. A wide range of electrodes can be selected depending on the target site, such as spoon electrodes, multielectrode arrays, customized defibrillator pads, caliper electrodes, plate electrodes, and nonpenetrating charged needles [85-86].

Hydrodynamic tail-vein injection (HTVI):

Hydrodynamic tail-vein injection (HTVI) is a gene delivery method involving the rapid injection of a large fluid volume—typically around 8–10% of the animal's body weight—into the bloodstream, most often in rodent models[87]. The rapid infusion generates transient hydrodynamic pressure due to the incompressibility of blood, temporarily disrupting both endothelial and parenchymal barriers. This mechanical effect enhances the uptake of nucleic acids and enables efficient delivery of CRISPR-Cas systems into hard-to-transfect tissues such as the liver, lungs, heart, and kidneys [88]. The technique has demonstrated significant success in preclinical studies, including liver cancer gene therapy and adenine base editing for hereditary tyrosinemia [89]. However, the necessity of high-volume, high-speed infusion can place substantial stress on hepatic tissues, limiting its applicability in clinical settings. Thus, improved safety profiles and optimized delivery protocols are essential for its future clinical translation [90].

Jet Gun:

This method utilizes a high-pressure, narrow stream of fluid to deliver therapeutic agents—including genetic material—directly into target tissues [91]. The intensity of the pressure applied plays a crucial role in ensuring uniform dispersion and effective penetration of the injected substance. While minor tissue trauma may occur, it is generally tolerable when effective gene expression and transfection are achieved [92]. For instance, in cardiac gene therapy, typical operational parameters include a jet velocity of 110 m/s, a distance of 20–25 cm, an injection volume between 100–500 µL, and an applied pressure of 150–250 kPa [93]. These adjustable parameters allow the optimization of tissue penetration depth while minimizing tissue damage. This technique, often referred to as the jet gun method, has been effectively used in a range of gene therapy approaches, including the delivery of genetic vaccines and suicide genes for antitumor treatments [94]. It has also been employed in treating genetic skin disorders [95]. Jet injection has demonstrated potential for gene delivery to multiple tissue types, including skin, mammary glands, muscle, and adipose tissue [96]. Though generally safe, mild side effects like minor bleeding at the injection site, localized inflammation, edema, or transient hyperthermia have been reported [97].

APPLICATION:

1. Application of CRISPR/Cas9 in Crop Quality Improvement
   CRISPR/Cas9 has been widely utilized in agricultural biotechnology to enhance crop quality traits, including yield, disease resistance, nutritional value, and stress tolerance. For instance, genome editing has been employed to develop rice and wheat varieties with improved grain quality and resistance to fungal pathogens [98].

2. Application of CRISPR/Cas9 for Biomedical Discoveries
   In biomedical research, CRISPR/Cas9 has accelerated gene function studies, drug discovery, and disease modeling. Its ability to induce precise gene knockouts or knock-ins has enabled researchers to study complex disease pathways *in vitro* and *in vivo* [99].

3. Therapeutic Application of CRISPR/Cas9 Technologies for HIV
   CRISPR/Cas9 offers promising strategies for targeting integrated HIV-1 proviral DNA within host genomes. Studies have demonstrated its potential in excising HIV-1 sequences from infected cells, representing a potential step toward functional cures [100].

4. Application of CRISPR/Cas9 in Cancer Treatment: A Future Direction
   In oncology, CRISPR/Cas9 is being explored for editing tumor-associated genes, enhancing T-cell immunotherapy, and generating personalized cancer models. Though still in preclinical stages, it holds significant promise for precise and durable cancer treatment modalities [101].

5. The CRISPR/Cas9 system is primarily applied in genome editing and transcriptional regulation, enabling targeted modification of DNA sequences and control of gene expression [102].

CONCLUSION

The CRISPR/Cas system has redefined the landscape of genetic engineering through its remarkable specificity, versatility, and adaptability. From its origins as a bacterial immune mechanism, CRISPR has evolved into a powerful genome-editing tool applicable in numerous biological and therapeutic domains. Advancements in Cas variants, delivery systems, and targeting mechanisms continue to improve its precision and reduce off-target effects. Viral vectors such as AAV, adenoviruses, and lentiviruses, alongside non-viral platforms like lipid nanoparticles and polymeric carriers, have expanded the reach of CRISPR into clinical and agricultural applications. Despite some challenges, including delivery efficiency and immune responses, CRISPR holds enormous promise for treating genetic disorders, improving crop traits, and developing personalized medicine. As research continues, CRISPR technology is poised to become a cornerstone in the future of molecular medicine and biotechnology.

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