CRISPR as a Tool for Targeted Drug Delivery: A Revolutionary Approach to Precision Medicine

Kunal Rathod[1] , Shravani Wankhade[2], Kartik Meghe[3] , Vihan Rajguru[4] Nilesh Mishra[5] , Vinod Thakare[6] , Tilottama Gatkine[7]

1. **Nagpur College of Pharmacy, Wanadongri, Hingna Road, Nagpur-441110**

ABSTRACT

The CRISPR-Cas system has emerged as a revolutionary force in genome editing, offering a level of precision, versatility, and efficiency that far outstrips traditional tools like ZFNs and TALENs. However, the transition from laboratory success to clinical reality hinges on a critical challenge: delivery. The safety and effectiveness of CRISPR therapies are entirely dependent on the ability to transport Cas enzymes and guide RNAs to the right cells; inefficient delivery risks causing off-target mutations, triggering immune responses, or failing to achieve therapeutic goals. This review outlines the fundamental mechanisms of CRISPR and its variants, while critically analyzing the limitations of current delivery methods. A major focus is placed on the promise of advanced nanocarriers such as lipid nanoparticles, polymeric systems, inorganic carriers, and extracellular vesicles which are designed to protect the genomic payload and enhance intracellular uptake. We further discuss how these delivery innovations are driving progress in treating cancer, genetic disorders, infectious diseases, and neurological conditions. Finally, we address the hurdles that remain, including immunogenicity and ethical concerns, and look ahead to the future of genome editing, which involves base and prime editors alongside AI-optimized smart nanocarriers**.**

Key Words: CRISPR-Cas9 System , Nanocarriers (Lipid, Polymeric, Inorganic) , Single Guide RNA (sgRNA) , Protospacer Adjacent Motif (PAM) , Ribonucleoprotein (RNP) , Off-Target Effects , Prime Editing (PE) , Cancer Therapy , Genetic Diseases

1. INTRODUCTION

1.1 Importance of Targeted Delivery

The foundational requirement for successful therapeutic gene editing using CRISPR-Cas components is their safe and effective transport into the nucleus of the target cells. Targeted drug delivery is crucial for overcoming key limitations inherent to CRISPR technology, such as insufficient tissue specificity, poor cellular uptake, potential immune activation, and the risk of off-target edits. For direct *in vivo* applications, delivery systems must precisely guide

the CRISPR cargo to diseased tissues while actively avoiding healthy organs, thereby minimizing unnecessary exposure to Cas nucleases. Achieving this high level of specificity is vital for reducing systemic immune responses and preventing unintended genetic modifications. An optimal carrier system should demonstrate a high affinity for its target cells, possess minimal cytotoxicity, ensure efficient intracellular trafficking, and facilitate the rapid clearance of CRISPR components once the editing is complete. These targeted delivery strategies are essential for enhancing both the safety and therapeutic effectiveness of CRISPR-based interventions, making them indispensable for successful clinical translation. [1]

1.2 Limitations of Traditional Systems

Before the advent of the CRISPR/Cas system, gene-editing relied on tools like zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). These earlier technologies presented several significant limitations, including low editability, a high off-target rate, high cytotoxicity, high cost, and extensive time and labor requirements. In sharp contrast, the CRISPR/Cas system is much simpler to design, less expensive, offers superior targeting efficiency, boasts a lower off-target rate, and exhibits reduced cytotoxicity compared to both TALENs and ZFNs. These clear advantages have quickly established the CRISPR/Cas system as an immensely potent tool for genome editing. [2]

1.3 Brief Introduction to CRISPR-Cas Technology

Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR-Cas9) is a rapidly evolving gene-editing technology that has garnered widespread attention. It is recognized as a third-generation gene-editing technology, surpassing older methods like ZFNs and TALENs in terms of flexibility, effectiveness, and precision, granting it greater potential for application.The CRISPR-Cas9 system is typically administered in one of three forms: DNA, messenger RNA (mRNA), or ribonucleoprotein (RNP), with each format having its own set of advantages and limitations. The specific choice of delivery form depends entirely on the selected delivery vector and the intended downstream application. Despite its power in genome editing, practical and technical obstacles, such as achieving efficient delivery, still need to be resolved before it can be widely adopted in clinical settings. The effective delivery of the CRISPR-Cas9 system remains essential for successful gene editing and subsequent clinical applications.[3]

1.4 Rationale for Integrating CRISPR with Nanocarriers

The integration of the CRISPR/Cas9 system with nanocarriers is vital for overcoming the challenges of efficient *in vivo* delivery, which is a prerequisite for its therapeutic use, particularly in cancer treatment. Current delivery methods, including both viral vectors and physical techniques, are often hampered by low editing efficiency in tumors, limited cargo size, and potential toxicity or immunogenicity. Nanocarriers, such as polymeric and lipid nanoparticles (LNPs), present a highly promising non-viral alternative, offering advantages like high compatibility, broad availability, and low cost. The core goal of this integration is to develop effective carriers for tissue-specific delivery of Cas9 and sgRNA, thereby markedly

improving both the targeting performance and safety profile, which is critical for transforming CRISPR/Cas9 into a reliable therapeutic agent.[4]

1.5 Objectives and Scope of the Review

This review aims to detail the necessity of targeted delivery for the CRISPR-Cas system in precision medicine. The scope encompasses an overview of CRISPR/Cas variants and their mechanisms, the limitations of traditional delivery methods, and a thorough exploration of modern nanocarriers (lipid, polymer, inorganic) and advanced cargo forms (RNP, mRNA, pDNA) used to improve safety and efficacy. It also examines applications in diseases and current challenges like off-target effects.

2. OVERVIEW OF CRISPR-Cas SYSTEMS

2.1 Historical background & Discovery

The discovery of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) unfolded gradually over several decades. In 1987, Yoshizumi Ishino first reported the observation of unusual repeat-spacer sequences in *E. coli*, though their biological function was unknown at the time. Substantial progress began in 1993 when Francisco Mojica started to characterize CRISPR loci across various microorganisms, and by the year 2000, he had defined their core structural features. In 2005, Mojica theorized that CRISPR functioned as a unique adaptive immune system in bacteria and archaea, noticing that many spacers closely resembled phage and plasmid DNA. In that same year, Alexander Bolotin identified Cas genes in *Streptococcus thermophilus*, including a large nuclease that was later named Cas9. His research group also recognized the Protospacer Adjacent Motif (PAM), a sequence crucial for DNA recognition. The immune function of CRISPR was confirmed experimentally in 2007, when Barrangou and colleagues demonstrated that the acquisition or deletion of viral spacers directly determined the bacteria's resistance to phages. Horvath's work further established that Cas9 is necessary for the interference stage. By 2008, van der Oost had identified CRISPR RNAs (crRNAs), while Marraffini and Sontheimer clarified that the CRISPR system specifically targets DNA. The pivotal discovery of tracrRNA in 2011 by Charpentier's team allowed for the formation of the crRNA-tracrRNA duplex that guides Cas9. This historical progression culminated in 2012 with successful programmable DNA cleavage *in vitro* and, in 2013, with the successful use of the system for genome editing in mammalian cells.[5]

2.2 Mechanism of CRISPR-Cas (Cas9, sgRNA, PAM)

The CRISPR-Cas9 system is a core component of the adaptive immune system found in archaea and bacteria, whose purpose is to defend against invasive nucleic acids. It is considered the most widely used type within the Class 2 CRISPR-Cas systems due to its simplicity, high efficacy, and ease of use. The system fundamentally consists of two components: the Cas9 endonuclease and a single guide RNA (sgRNA).

- Cas9 Endonuclease: The Cas9 protein functions as a pair of molecular scissors, capable of cleaving the double strands of DNA. It contains two nuclease domains, RuvC and HNH. The RuvC domain is responsible for cleaving the non-target DNA

strand, while the HNH domain cleaves the complementary (or targeted) DNA strand; together, they create a double-strand break (DSB).

- Single Guide RNA (sgRNA): The sgRNA is the component responsible for recognizing the specific target DNA sequence. It is engineered as a single strand with two critical segments: a duplex RNA structure at the 3' end that binds the Cas9 protein, and a guide sequence at the 5' end that binds the target DNA sequence. The sgRNA's role is to guide the Cas9 protein to a precise sequence within the genome.
- Protospacer Adjacent Motif (PAM): The sgRNA directs Cas9 to cleave the DNA at a specific site, which is located three base pairs upstream of a "NGG" PAM sequence. The selection of the target DNA fragment, or protospacer, is strictly dependent on the presence of this PAM.

Once the sgRNA-Cas9 complex binds to the target DNA and creates a DSB, the cell activates its DNA repair mechanisms. This repair occurs through one of two main pathways: non-homologous end joining (NHEJ), which often introduces small insertions or deletions (indels) leading to gene knockout, or homology-directed repair (HDR), which requires a donor DNA template to achieve precise gene editing. [6,7]

2.3 CRISPR variants (Cas9, Cas12, Cas13, BE, PE)

The CRISPR/Cas system has evolved into a versatile genome editing toolbox, primarily classified into two distinct classes. Class 2, which encompasses Cas9, Cas12, Cas13, and Cas14, is the most frequently used in gene therapy because its effector function is carried out by a single protein.

Cas Proteins

- CRISPR/Cas9: This is the most widely applied gene editor. The Cas9 protein utilizes a dual-RNA complex of crRNA and tracrRNA, often engineered as a single guide RNA (sgRNA), to target and cleave double-strand DNA (dsDNA). Cleavage occurs typically three base pairs upstream of a PAM and results in blunt ends. The subsequent repair is mediated by either NHEJ or HDR.
- CRISPR/Cas12 (Cpf1): Unlike Cas9, the Cas12a system only requires the crRNA for guidance and recognizes a 5' TTTV 3' NPAM sequence to cleave dsDNA, which results in sticky ends. Cas12a has a distinct ability to indiscriminately cleave single-stranded DNA (ssDNA) after target recognition, a feature that is leveraged in diagnostic tools.
- CRISPR/Cas13: This system is unique because it specifically targets and cleaves single-stranded RNA (ssRNA). Cas13a functions with crRNA alone and, once activated upon target recognition, leads to the collateral cleavage of other nearby RNAs, although this collateral activity has not been clearly observed in eukaryotes.
- CRISPR/Cas14: Discovered in archaea, Cas14 is a smaller protein that targets and cleaves ssDNA without the requirement of a PAM sequence, offering high specificity for ssDNA.

Advanced Variants:

- Base Editing (BE): This is a precision technique designed to make single base changes (point mutations) without inducing a double-strand break (DSB). It involves linking a catalytically impaired Cas9 variant (such as a nickase or dead Cas9, dCas9) to a deaminase enzyme. This fused complex guides the deaminase to the target site, where it chemically alters a single base.
- Prime Editing (PE): This advanced method also avoids creating DSBs but has the versatility to introduce substitutions, insertions, or deletions. It utilizes a Cas9 nickase fused to a reverse transcriptase enzyme, along with a specialized prime editing guide RNA (pegRNA). The pegRNA guides the system to the target site, and its extended sequence serves as a template, allowing the reverse transcriptase to directly synthesize and insert the new DNA sequence.[2,8]

2.4 Advantages over other gene-editing tools

The CRISPR-Cas9 system has rapidly become the most widely adopted gene-editing technology due to its distinct benefits over other methods, such as ZFNs and TALENS.

Key advantages include:

- Ease of Design and Cost-Effectiveness: CRISPR-Cas9 is notable for the simplicity of designing its targeting components and its low associated cost. This is a significant improvement over ZFNs and TALENS, which are known for their complex designs and higher expense.
- High Efficacy: The technology delivers a high level of efficacy in its gene editing capabilities. This stands in contrast to the comparatively lower efficiency and limitations observed in other tools like ZFNs and RNA interference (RNAi).
- Broad Applicability: CRISPR/Cas9 is a highly flexible platform that can be successfully implemented across a wide range of organisms and cellular populations, including human cells.
- Reversible Gene Regulation: While standard CRISPR/Cas9 produces permanent changes, its dead Cas9 (dCas9)-based variants (CRISPRa/CRISPRi) offer the unique advantage of reversible gene expression alteration. This quality is also shared with Cas13-mediated RNA editing/antisense oligonucleotides but is not possible with the permanent editing methods of ZFNs or TALENS.[8]

3. DRUG DELIVERY SYSTEMS IN MODERN THERAPEUTICS

3.1 Principles of Targeted Drug Delivery

The foundational principle of novel drug delivery systems (NDDS) is to ensure the sustained and controlled release of therapeutic agents, a strategy intended to enhance efficacy while mitigating the limitations of conventional treatments. For a drug to achieve its optimal

therapeutic effect, it must reach the target tissue in the correct amount and at the right time, all while minimizing systemic toxicity and adverse reactions. Treatment failure in many diseases often stems from inefficient delivery methods, as a drug's pharmacological action is heavily influenced by its administration and transport. Because every drug has a defined therapeutic window, concentrations falling outside this range can either lead to toxicity or reduced effectiveness. Consequently, designing an efficient and precise drug delivery system (DDS) remains a major challenge.

Drug targeting involves directing drugs specifically to desired biological sites, which can be accomplished through either passive or active mechanisms. Targeted delivery can be enhanced by attaching bioactive molecules to carriers, which include systems such as liposomes, biodegradable polymers, implants, monoclonal antibodies, and particulate systems. Nanotechnology-based DDS further improve delivery by enabling sustained release, enhanced diffusion, and extensive surface area interactions. Overall, a DDS is composed of the drug formulation itself, the chosen method of administration, and the mechanism that controls the drug's release.[1,9]

3.2 Nanocarriers

Nanocarriers provide a promising non-viral approach for delivering the CRISPR/Cas9 gene-editing system, which currently faces hurdles due to its low intracellular delivery efficiency. The utilization of nanocarriers, such as liposomes, polymers, and inorganic nanoparticles, represents a critical advancement for gene delivery in therapeutic applications.

Types of Nanocarriers for CRISPR/Cas9 Delivery:

- Lipid-Based Nanoparticles (LNPs):
    - Lipid nanoparticles are classic delivery systems for transferring nucleic acids. They form a complex with negatively charged nucleic acids via host-guest and electrostatic interactions, facilitating uptake by endocytosis. LNPs are essential for protecting nucleic acids (plasmids or mRNAs) from nuclease degradation.
    - Liposomes are small, spherical manufactured vesicles composed of cholesterol and safe, natural phospholipids. Their size and amphiphilic properties make them efficient drug transport vehicles.
    - Hybrid exosomes, created by fusing exosomes with liposomes, can efficiently encapsulate large CRISPR/Cas9 expression plasmids.
    - Lipid Nanoparticles (LNPs), including cationic liposomes and other lipid nanoparticles, are widely used. Modified LNPs have been developed to enhance delivery; for example, some formulations containing ionizable lipids and permanent cationic supplements (like DOTAP) can preserve the integrity of the Cas9 ribonucleoprotein (RNP) and effectively target specific tissues, such as the liver and lungs, upon intravenous injection. Biodegradable lipid-

like nanoparticles (LLNs) have also been engineered to deliver Cas9 mRNA *in vitro* and *in vivo*.

- Polymer-Based Nanoparticles:
    - Cationic polymer carriers, such as polyethyleneimine and chitosan, offer chemical diversity and flexible structural designs for delivering various types of nucleic acids, including plasmid DNA and mRNA.
    - Similar to lipid carriers, polymer nanoparticles traverse the cell membrane via endocytosis and shield the payload from nuclease degradation and the immune response.
    - Researchers have developed sophisticated systems, including dual-targeting polymer/inorganic hybrid nanoparticles, capable of encapsulating CRISPR/Cas9 plasmids and efficiently delivering them to the nucleus of tumor cells.
    - Multistage delivery polymer nanocarriers (MDNP) can be designed with a core-shell structure using a responsive polymer that reacts to the slightly acidic environment of a tumor, enabling tumor-targeted delivery of the CRISPR/Cas9 system.
- Inorganic Nanoparticles:
    - Inorganic nanoparticles also function as effective delivery platforms, often utilized for RNP delivery.
    - Gold Nanoparticles (AuNPs) are a newer class of RNP delivery vehicles. They can be cross-linked with sulfhydryl substances and have adjustable surface charge and hydrophilicity. For instance, cationic arginine-functionalized gold nanoparticles (ArgNPs) have been successfully used to deliver chemically modified Cas9 protein and sgRNA.
    - CRISPR-Gold technology, which involves coupling thiol-modified oligonucleotides to AuNP surfaces followed by cationic polymer encapsulation, has been used to repair the mutant dystrophin gene in mice.
- Extracellular Vesicles-Based Delivery (Exosomes):
    - Exosomes, a type of extracellular vesicle, are considered the most promising non-viral nanocarrier due to their naturally high biocompatibility and low immunogenicity.
    - Exosomes can efficiently deliver the CRISPR/Cas9 system, carrying Cas9 protein, gRNA, plasmids, or mRNA.
    - They help to reduce the risks of off-target effects and genome integration because the Cas9 proteins and gRNA are rapidly degraded inside cells.

- Various engineered exosomes have been developed to enhance loading and delivery efficiency, such as those that fuse the Cas9 protein with exosomal membrane proteins or utilize RNA-binding protein domains for Cas9 mRNA loading.

Nanotechnology for CRISPR/Cas9 delivery is a rapidly advancing field, providing a crucial set of tools for overcoming the significant challenge of efficient intracellular delivery required for gene editing. [9,10,11]

3.3 Ideal criteria for Nanocarriers

Ideal nanocarriers for CRISPR delivery must successfully address critical challenges, with the ultimate objective of achieving efficient and safe intracellular delivery.

Key criteria include:

- Biocompatibility and Low Cytotoxicity: Nanocarriers must exhibit minimal adverse effects on cell viability. The cationic hyper-branched cyclodextrin-based polymer (Ppoly) system demonstrated this, with cell viability remaining above 80% even at high concentrations. This represents a critical advantage over conventional, often more toxic, lipid-based transfection reagents.
- Targeting and Specificity: The carriers must ensure efficient cellular uptake and enhance the stability of the cargo. The positive surface charge of Ppoly aids in strong complexation with the negatively charged RNP, a feature essential for efficient cellular internalization.
- Stability: The delivery system must ensure serum stability and reduce degradation. Cyclodextrin-based nanocarriers, such as Ppoly, offer enhanced stability and superior loading capacity. The nanosponge architecture further improves both stability and biocompatibility.[12]

4. CRISPR-BASED TARGETED DRUG DELIVERY APPROACHES

The CRISPR/Cas9 system can be introduced into host cells in three primary forms, which are collectively referred to as "cargo": plasmid DNA (pDNA), messenger RNA (mRNA) plus single guide RNA (sgRNA), or the ribonucleoprotein (RNP) complex (Cas9 protein complexed with sgRNA). The selection of the cargo form and its delivery method directly influences the time required before gene editing takes place and the corresponding likelihood of off-target effects.

4.1 Delivery Forms of CRISPR/Cas9 Cargo:

1. Plasmid DNA (pDNA)

The pDNA encoding the Cas9 endonuclease and sgRNA is the most commonly used form because of its stability, ease of construction, and flexibility of preparation. Once successfully delivered into the cell's nucleus, it undergoes transcription and translation to express the functional Cas9 endonuclease. This multi-step process means that detectable genomic editing events take 24-48 hours to appear. A major drawback is that pDNA can replicate and has the

potential for undesirable stable integration into the host genome, leading to long-term Cas9 expression and an increased chance of off-target gene editing.

2. Messenger RNA (mRNA)

Cas9 mRNA complexed with sgRNA is simpler to introduce and offers quicker expression than pDNA because it bypasses the nuclear delivery requirement for transcription and can begin translation instantly. This approach alleviates the risk of insertional mutagenesis and reduces the chance of off-target effects due to the mRNA's short-lived presence in the cell. However, mRNA is inherently unstable compared to pDNA and is highly susceptible to RNase-mediated degradation.

3. Ribonucleoprotein (RNP)

The RNP complex, which consists of the purified Cas9 protein directly complexed with sgRNA, provides the most rapid gene editing because it bypasses both the transcription and translation steps. Editing events can be detected within 1 hour of delivery. RNP-based approaches demonstrate reduced off-target effects compared to pDNA because the Cas9 endonuclease has a short-lived, transient presence. A challenge is that the high molecular weight of the Cas9 protein and the difficulty of transporting a negatively charged protein complex across the cell membrane can make RNP loading and delivery challenging, requiring more optimization when formulating into nanoparticles. These three forms (pDNA, mRNA, RNP) ultimately converge into a single functional pathway: pDNA leads to mRNA, and mRNA leads to the final RNP complex, which is then imported into the nucleus to perform genome editing.[13,14]

4.2 Delivery Carriers for CRISPR-Cas Components

The successful therapeutic use of CRISPR-Cas systems for genome editing demands the efficient delivery of the CRISPR components the Cas enzyme and guide RNA (gRNA) into the nucleus of targeted cells. Various delivery carriers, which are broadly categorized as viral and non-viral, have been investigated to overcome the challenge of transporting these large, charged molecules across the cell membrane while protecting them from degradation.

Viral Vectors (e.g., AAV)

Viruses are often used in laboratories for Cas9 expression because they have naturally evolved for highly efficient cell invasion.

- Adeno-associated virus (AAV) is the most common viral vector employed for Cas9 delivery.
- Viral particles are engineered by replacing their replication genes with a therapeutic transgene, which allows them to enter a host cell and induce transgene expression without replicating or spreading.
- Different AAV serotypes can be selected to enhance targeted delivery to a specific organ, as each serotype shows a preferential delivery efficiency to certain cell types.

- A major limitation is the physical size capacity of the virus; the maximum capacity of an AAV is approximately 4.7 kB. This size restriction makes packaging the large Cas9 gene, its promoter, and the gRNA challenging. Smaller Cas9 orthologs, such as *Staphylococcus aureus* Cas9 (SaCas9), are better suited for packaging in viral particles.
- Concerns for clinical use include the risk of the transgene disrupting a vital gene in the target genome (insertional errors) and the potential for a more persistent expression time course, which may increase the opportunity for off-target editing events compared to transient formats.

Non-Viral Delivery: Chemical Approaches

Non-viral delivery eliminates the size limitations and significantly reduces the risk of insertional errors associated with viral vectors, often allowing for tighter control over the dose, duration, and specificity of delivery. Chemical delivery utilizes complementary molecules to help the CRISPR cargo bypass cellular barriers and protects it from degradation.

- Lipid-Based Carriers (LNPs):
    - Lipid-based carriers are popular and highly efficient for delivering CRISPR DNA, mRNA, or ribonucleoproteins (RNPs).
    - Formation: Lipids are amphiphilic molecules that spontaneously form nanoparticles in an aqueous solution, which can either encapsulate the payload or complex with it.
    - Cationic Lipids: Synthetic cationic lipids are especially effective at forming a complex with negatively charged payloads like DNA, mRNA, or the Cas9 RNP complex (due to the negatively charged gRNA molecule).
    - Protection and Targeting: Lipid carriers can shield the payload from enzymatic degradation and immunological responses. This protection can be enhanced by modifying the nanoparticles with long-chain PEG molecules. They can also be modified with active targeting moieties to improve tissue-specific delivery.
- Polymeric Carriers (PLGA, PEI):
    - Encapsulation within polymeric carriers is another common approach due to the wide variety of available polymers.
    - Materials: Polymers can be synthesized from natural monomers, such as sugars (e.g., chitosan), or synthetic monomers, like poly-caprolactone (PCL) or poly-lactic/glycolic acid copolymers (PLGA).
    - Protection and Targeting: Similar to lipid encapsulation, polymeric carriers can protect and conceal the cargo from *in vivo* degradation pathways and can also be functionalized with active targeting moieties.

- Toxicity: A drawback is that polymeric nanoparticles can result in high toxicity, particularly with synthetic monomers, which can reach high concentrations upon degradation.

- Inorganic Nanoparticles (e.g., Gold):
  - Macromolecules can be delivered by forming a complex with nanoparticles made of inorganic materials such as gold, silica, and carbon nanotubes.
  - Advantages: Inorganic nanoparticles are extremely colloidally stable, which minimizes the risk of early clearance, and their size, composition, and distribution can be precisely controlled.
  - CRISPR-Gold: The use of gold nanoparticles for Cas9 RNP delivery is growing in popularity and is considered a promising approach for *in vivo* RNP delivery. Gold nanoparticle-mediated Cas9 RNP delivery has successfully initiated gene editing and homology-directed DNA repair (HDR) *in vivo*.

- Chemical Modification (CPPs, NLSs):
  - Delivery can be accomplished by directly chemically modifying the proteins and nucleic acids without the need for encapsulation.
  - Cell-Penetrating Peptides (CPPs): These are short peptide sequences capable of crossing the cell membrane. Conjugating Cas9 protein and gRNA with CPPs has been shown to enhance protein delivery. However, CPPs do not provide the protection from degradation or the cell-type specific targeting that encapsulation methods offer, often requiring them to be combined with other techniques.
  - Nuclear Localization Sequences (NLSs): These naturally occurring sequences tag proteins for transport into the nucleus. They are typically poly-arginine or poly-lysine chains conjugated to the protein surface and stimulate nuclear import signals to allow passage through the nuclear membrane. Because Cas9 must reach the nucleus to function, all Cas9 applications include an NLS.[15]

4.3 Targeting Strategies for CRISPR/Cas9 Delivery

Effective delivery of the CRISPR/Cas9 system to the correct target site is essential to fully realize its gene-editing potential. Enhancing its targeting and delivery capabilities is crucial for widespread clinical applications. A variety of strategies, including the use of ligands, aptamers, antibodies, and promoters, are employed to achieve this necessary specificity.

- Ligands, Aptamers, and Nanocarriers:
  - Ligands can be used to modify non-viral vectors to improve delivery specificity. For example, ligand-modified chitosan nanoparticles have significantly enhanced the specificity of the CRISPR/Cas9 system.

- Extracellular vesicles (EVs) are natural carriers that can transport exogenous substances to specific cells or tissues for targeted delivery. Exosomes derived from tumor cells are suitable natural carriers for delivering CRISPR/Cas9 to tumor cells, and modifying their surface can further boost their targeting ability.
- The high-capacity adenovirus (HCAdV) can be utilized to concurrently encapsulate multiple genes, such as fluorescent or luminescent reporters, alongside CRISPR/Cas9 components in a single vector, which improves transduction efficiency and reduces host immune responses compared to using multiple vectors.
- Aptamers, such as the MUC1 aptamer, can be integrated into DNA nanostructures to help the loaded cargo escape from lysosomes, thereby assisting in the targeted delivery of the Cas9/sgRNA complex.

- Stimuli-Responsive Systems and Promoters:
  - Stimuli-responsive nanoparticles, which can be triggered by internal signals like pH, ATP, or glutathione, or by exogenous stimuli like light and magnetic fields, enable more precise gene editing and targeted delivery.
  - Promoters are factors that contribute to transfection efficiency in lentiviral and other vector systems. CRISPR-based gene activation (CRISPRa) can selectively target promoters or enhancers in a tissue/cell type-specific manner to upregulate gene expression. [16]

5. APPLICATIONS OF CRISPR IN TARGETED DRUG DELIVERY

5.1 Cancer therapy

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 system is a robust gene-editing technology that has revolutionized cancer therapy, particularly in the field of immunotherapy. Due to its simplicity, high fidelity, and potential for multi-target editing, CRISPR-Cas is preferentially used over earlier nuclease-based systems like ZFNs and TALENS.

Applications in Adoptive Cell Transfer (ACT):

The primary application of CRISPR in cancer therapy is the engineering of T-cells for Adoptive Cell Transfer (ACT) immunotherapy. This is crucial for constructing allogeneic universal T-cells that can specifically target tumor cells with enhanced function, durability, and minimal side effects.

- CAR/TCR T-cell Enhancement: CRISPR-Cas9 is used to disrupt or edit specific genes in T-cells, which are the fundamental materials for engineered T-cells, in order to enhance their function and customize their properties. The technology facilitates the creation of Chimeric Antigen Receptor (CAR) T-cells or the modification of T-cell Receptor (TCR) T-cells.

- Preventing Rejection and Exhaustion: To generate "universal" T-cells, CRISPR is employed to knock out the endogenous TCR to prevent potential graft versus host disease (GVHD) and simultaneously silence human leukocyte antigen (HLA) expression to prevent immediate host immune rejection. Furthermore, blocking inhibitory receptors, known as immune checkpoints (e.g., PD-1, CTLA-4, LAG-3, FAS), using CRISPR postpones T-cell exhaustion and reinforces their anti-tumor activity.
- Precise Knock-in: CRISPR-mediated Homology-Directed Repair (HDR) can be used to precisely insert the CAR or recombinant TCR cassette into a desired locus, like the TRAC locus, which also simultaneously knocks out the endogenous TCR. This method minimizes heterogeneity and eliminates the mutation probability commonly seen in vector integration methods.

Research and Screening:

Beyond direct therapeutic editing, CRISPR is extensively used in preclinical studies and genome-wide screening to advance cancer immunotherapy.

- Target Discovery: CRISPR screening identifies key regulatory elements of immune response pathways, such as Ptpn2 (protein tyrosine phosphatase non-receptor type 2), SOCSI, and CBLB, whose knockout can improve the proliferation and anticancer capability of T-cells.
- Cancer Modeling: The system is vital for constructing cancer models, identifying and validating novel drug targets and biomarkers, and elucidating drug resistance mechanisms.
- Epigenetic and Virotherapy: CRISPR can be exploited for epigenome editing and in oncolytic virotherapy by modifying oncolytic viruses, such as constructing IL-15-expressing herpes simplex virus II to increase anti-tumor activity. [17,18,19]

5.2 Genetic diseases

The application of CRISPR technology in the treatment of genetic diseases has expanded dramatically, moving past simple gene knockout to include sophisticated genome engineering strategies. This advancement enables more precise and effective therapeutic approaches for both monogenic disorders and diseases driven by complex factors.

Targeted Gene Editing:

The foundational application of CRISPR-Cas systems involves using the Cas protein (such as Cas9) guided by RNA to create double-strand breaks (DSBs) in the genome. The subsequent repair by the Non-homologous end joining (NHEJ) pathway often results in insertions and deletions (indels), effectively knocking out harmful mutant genes. This method has been used in preclinical studies and clinical trials for monogenic disorders. For instance, to treat the rare, fatal neuropathy hereditary transthyretin amyloidosis (hATTR), a CRISPR-based therapy was developed to deliver Cas9 and a guide RNA (sgRNA) to target the TTR gene. The resulting DSB induces a frameshift mutation, effectively silencing the mutant gene.

Precision Gene Correction:

To achieve precise gene correction beyond random indel formation, two advanced CRISPR technologies, Base Editing and Prime Editing, were developed:

- Base Editors (BEs): These systems fuse a nuclease-dead Cas protein (dCas) or a nickase Cas protein (nCas) to a deaminase enzyme.
- Cytosine Base Editors (CBEs) convert C to T (via a C-to-U conversion read as T upon replication).
- Adenosine Base Editors (ABEs) convert A to G (via an A-to-I deamination resolved as G).
- BEs are actively being investigated for diseases like phenylketonuria (PKU) and to correct a nonsense mutation in a mouse model of sickle cell disease.
- Prime Editors (PEs): These systems fuse an nCas9 to a reverse transcriptase (RT) and use a long prime editing guide RNA (pegRNA). PEs are versatile, capable of introducing targeted insertions, deletions, and all 12 types of base-to-base conversions, offering a more comprehensive approach to correcting complex pathogenic mutations. They have been used to correct mutations causing sickle cell disease and Tay-Sachs disease.

Non-Coding and Epigenetic Modification:

CRISPR tools are also used to target non-coding regions to modulate gene expression, which is essential since many diseases are driven by mutations in regulatory elements like promoters or enhancers.

- Gene Regulation (CRISPRI/CRISPRa): Nuclease-dead Cas proteins (dCas) can be fused to transcriptional repressors (like KRAB for CRISPR interference (CRISPRi) or activators (like VP64 for CRISPR activation (CRISPRa)) to achieve targeted gene downregulation or upregulation without altering the genetic code. This approach is currently being studied for conditions such as chronic pain and obesity.
- Epigenetic Editing: Fusing dCas to epigenetic modulators (e.g., DNA methyltransferases or histone modifiers) enables persistent and heritable changes to gene regulation, offering potential long-term therapeutic benefits for diseases like Parkinson disease and fragile X syndrome.

These diverse applications demonstrate that CRISPR systems are essential for engineering the human genome to create potential curative therapies for a wide range of diseases.[20,21]

5.3 Infectious diseases

CRISPR has shown promising potential in the fight against infectious diseases, specifically HIV infection and malaria, by targeting and modifying genetic material.

- HIV Infection: For HIV infection, researchers have used CRISPR to delete the viral DNA that has been integrated into the human host cell. These studies show promise for generating genetically repaired tissue to prevent viral DNA function. Similar

strategies have involved targeting multiple sites in the viral DNA to reduce the chance of the virus escaping or developing resistance to the primary treatment. The CRISPR system has also been used to modify the HIV co-receptors CCR5 and CXCR4, which is a hypothetically safe method for protecting CD4+ cells from HIV-1 infection. Eliminating the HIV genome from eukaryotic cell lines using CRISPR has yielded promising treatment strategies.

- Malaria Resistance: The CRISPR system has also been applied to create mosquitoes that can transfer resistance to malaria within their own species. This involves modifying the DNA of female *Anopheles* mosquitoes, a major parasite carrier, by injecting edited antibodies programmed to attack the malaria parasites. *In vitro* matings between modified and unmodified mosquitoes have shown a 99.5% promise with this technique. However, this application is still nascent and requires further analysis and experimental design, with all risk factors needing validation before extensive use in nature.[5,22]

5.4 Neurological Disorders and BBB Transport

The CRISPR-Cas9 system holds significant promise for the treatment of neurological disorders due to its capacity for precise gene editing. Numerous animal models have been developed using CRISPR/Cas9 to study neurological conditions, enabling scientists to investigate the pathophysiology and develop therapeutic interventions.

- In pigs, models for Parkinson's disease (PD) have been created by generating DJ1 knock-out, PARK2/PINK1 double knockout, and Parkin/DJ-1/PINK1 triple knockout lines using CRISPR/Cas9 and TALENS. A Huntington's Disease (HD) knock-in pig model was also generated, showing selective neurodegeneration that mimics the human disease.

- In rodents, a Parkinson's disease rat model was created by destroying the Tyrosine Hydroxylase (TH) gene in dopaminergic neurons via intracranial injection of AAV vectors expressing Cas9 and gRNA.

A major hurdle for gene editing in the brain is the delivery of the CRISPR/Cas9 components. The blood-brain barrier (BBB) limits the passage of therapeutic agents to the brain. While the provided text discusses various delivery methods (viral and non-viral) and acknowledges the general challenge of delivery, it does not explicitly discuss using CRISPR/Cas9 to specifically enable or study transport across the blood-brain barrier (BBB). Overcoming these delivery barriers remains a critical factor for widespread *in vivo* application.[22]

5.5 Regenerative medicine

The CRISPR-Cas system shows promise in gene therapy, with applications that significantly overlap with regenerative medicine, particularly in the editing of various cell types, including stem cells.

Targeting Muscular Dystrophy and Blood Disorders:

- In the treatment of Duchenne muscular dystrophy (DMD), CRISPR/Cas9 technology is planned to restore the disrupted DMD reading frame through permanent exon skipping/reframing/deletion, which can lead to dystrophin restoration. *In vitro* removal of exons 45-55 has successfully resulted in the restoration of dystrophin protein synthesis. Furthermore, in mouse models, CRISPR successfully removed the defective part of the gene, allowing the animals to produce a major muscle protein, which is promising for future DMD treatments.
- For inherited blood disorders like sickle cell anemia and β-thalassemia, CRISPR has been used to modify stem cells' DNA by deleting the BCL11A gene. This gene deletion removes the suppression of fetal hemoglobin production, allowing the stem cells to produce sufficient fetal hemoglobin to overcome the effect of the defective adult hemoglobin.

Cell-Based Therapy:

For Haemophilia B, scientists successfully modified human induced pluripotent stem cells (iPSCs) derived from patients by inserting the complete F9 human cDNA using CRISPR/Cas9. Upon transplantation into mice, these modified iPSCs secreted human FIX, a promising result for future studies. Additionally, the technology was tested on a mouse model for Hereditary tyrosinemia type I (a genetic liver disease) to correct a mutation, suggesting that CRISPR/Cas9 can be used in adult animals and humans to correct genetic diseases.[23]

6. EVALUATION & CHARACTERIZATION TECHNIQUES

6.1 Particle size, PDI, zeta potential

The size, polydispersity index (PDI), and zeta potential of Lipid Nanoparticles (LNPs) are crucial parameters that influence the efficiency and safety of CRISPR/Cas9 delivery.

- Particle Size and Polydispersity Index (PDI): Particle size is a factor in determining the biodistribution of LNPs after intravenous injections. Nanoparticles smaller than 10 nm in diameter may be removed by the kidneys, while those larger than 200 nm can accumulate in the liver and spleen due to the activation of the complement system. However, LNPs $\approx$ 150 nm in size are capable of escaping the fenestrated capillaries in the liver. PDI is a measurement of particle size distribution. Optimized LNP formulations aim for an appropriate size for optimal CRISPR/Cas9 delivery and stability.
- Zeta Potential: The zeta potential is a fundamental parameter of particle stability, representing the magnitude of the LNP surface charge which influences electrostatic interactions between particles. The overall charge of the LNPs plays a role in their *in vivo* biodistribution. For instance, altering the ratio of lipids like permanently cationic DOTAP and the helper lipid DOPE can significantly impact the particle's zeta potential.

Ultimately, the overall charge, particle size, lipid ratio, and receptor-mediated ligand or antibody conjugations underscore the versatility of LNPs as a delivery platform that can achieve desired target specificity *in vivo*. [24]

6.2 Loading Capacity and Encapsulation Efficiency

Ideal delivery vehicles for protein cargo must achieve high packaging efficiencies. The study used SDS-PAGE analysis of degraded CRISPR-Gels to assess the amount of ribonucleoprotein (RNP) lost during the synthesis process. The encapsulation efficiency was defined as the relationship of the total input Cas9 to the total Cas9 found in the resulting nanogel solution. This magnitude is generally considered suitable for RNP delivery. The presence of a dominant band matching the electrophoretic mobility of pure, unencapsulated Cas9-RNP standards after reduction suggests the protein remains intact. This supports the theory that RNP can be immobilized by nanogels and protected from degradation.

For the cargo, a larger *S.p.* Cas9 protein (6xHis-MBP-4xNLS-Cas9-sfGFP) was used, with a total molecular weight of 237 kDa. This is larger than normal *S.p.* Cas9 (160 kDa) and comparable to common base editors (around 220 kDa), indicating that the encapsulation capability extends to these larger, multifunctional proteins. The mechanism of immobilization is likely sterical immobilization by the polyglycidol (PG-SH) nanogel mesh, as covalent bonding is not probable.[25]

6.3 In Vitro/In Vivo CRISPR/Cas Delivery

In Vitro Transfection

Initial therapeutic applications of CRISPR/Cas were often performed ex vivo (on cells outside the body) using established methods like microinjection or electroporation, and newer methods such as TRIAMF and iTOP. These methods are extensively used in vitro to study CRISPR/Cas effects, as they are economical and easy to implement on cell lines.

- Microinjection is effective for generating knockout animals by injecting CRISPR components into zygotes, but it requires individual cell manipulation.
- Electroporation forms pores in cell membranes with high voltage to transfect cells *ex vivo*, but it can be highly toxic.
- TRIAMF (induction of transmembrane internalization assisted by membrane filtration) and iTOP (induced transduction by osmocytosis and propane betaine) are newer techniques developed to deliver ribonucleoproteins (RNPs) with potentially less cytotoxicity.

In Vivo Delivery

For direct in vivo application, delivery systems are necessary to target CRISPR/Cas components to specific tissues or cells in the human body without causing immune activation or high off-target effects. This can be achieved intravenously or through local administration (e.g., intramuscularly). Local administration provides a high dose in the target tissue for a high likelihood of gene editing, while intravenous administration can reach a wider, systemic

target. However, the feasibility of using these direct methods in vivo is limited, and other methods are required for direct in vivo delivery.[26]

6.4 Imaging and Biodistribution of Lipid Nanoparticles (LNP)

Lipid nanoparticles (LNPs) are crucial for the systemic delivery of siRNA, as naked siRNA is rapidly degraded and eliminated by the kidneys.

Biodistribution and Uptake:

Systemically administered naked siRNA typically accumulates in the liver and kidney, failing to reach the disease site. LNP systems, however, represent the most advanced technology for systemic delivery of siRNA. LNP-siRNA systems depend on the lipoprotein pathway for effective uptake in the liver (hepatocytes).

- Specifically, the apolipoprotein ApoE mediates the hepatic uptake of ionizable LNP-siRNA. This uptake is reduced in ApoE-deficient mice, but efficacy can be restored by conjugating N-acetylgalactosamine (GalNAc) to the PEG lipid, which targets the asialoglycoprotein receptor on the hepatocyte surface.
- For disease sites like tumors, the Enhanced Permeation and Retention (EPR) effect is key for biodistribution. Long-circulating liposomes preferentially escape the "leakier" neovasculature at tumor sites, and impaired lymphatic drainage retains them in the interstitial space, significantly increasing drug delivery to the tumor.

Imaging and Theranostic Systems

The LNP carrier system can be leveraged to create dual-functionality, or "theranostic," systems by co-encapsulating metallic nanoparticles with siRNA.

- Metallic nanoparticles can serve as contrast agents for bioimaging. For example, gold nanoparticles are good absorbers of X-ray radiation and can enhance contrast during X-ray imaging of tumors.
- An LNP carrier system has the potential to improve the typically poor accumulation of metallic nanoparticles in tumors by taking advantage of the EPR effect.
- Co-encapsulation of siRNA with a metallic nanoparticle allows for both the bioimaging of the tumor and the silencing of the target gene for therapeutic purposes.[27]

6.5 Gene Editing Efficiency Assays

Several methods have been reported for detecting mutations induced by the CRISPR/Cas system at targeted loci in various organisms. These include polymerase chain reaction (PCR)-based assay, T7 Endonuclease I (T7EI), high-resolution melting curve analysis (HRM), and Next-Generation Sequencing (NGS)-based methods. However, these traditional methods are generally semiquantitative and often struggle with accurately quantifying editing frequency, particularly at very low levels in complex polyploid plant genomes or when detecting processed food samples containing low initial concentrations of DNA.

A more powerful tool is the droplet digital PCR (dPCR)-based method. dPCR is an ultrasensitive technology that provides absolute nucleic acid quantification without the need for a standard curve. The study reported developing a duplexed dPCR-based method for the detection and evaluation of gene-editing frequencies in plants. Compared to qPCR and NGS-based methods, the dPCR assay showed a lower limit of detection (LOD) for the editing frequency (detecting as low as 0.1% mutant template) and a better linear relationship with the expected editing frequency (Pearson's $R^2 > 0.999$). This method is applicable to polyploid plants and is especially useful for detecting gene-edited food samples with low DNA concentration.[28]

7. CHALLENGES AND LIMITATIONS

7.1 Off-Target Effects

The ability to manipulate genetic targets with CRISPR-based drugs introduces the practical challenge of ensuring the drug enters only the desired cell without causing unwanted outcomes. This is a major hurdle, and the risk of unintended genomic modifications is specifically highlighted by FDA guidelines. In the context of DNA-editing CRISPR systems, the duration of nuclease expression is a primary concern. Longer nuclease expression from an AAV vector DNA could lead to off-target editing because the editing effect (such as NHEJ-mediated loss of function, base edits, or insertions) is permanent, even in rapidly dividing cells. For instance, lentiviral vectors are generally considered a disadvantage for DNA-editing CRISPR drugs because their integration into the genome would cause an active nuclease to persist for long periods, thereby increasing the odds of editing at off-target loci.

Conversely, in an RNA nuclease-based therapy, the enzyme would be expressed for a longer period, as the change produced is not permanent. The concern about persistent expression suggests that vectors carrying an RNA cargo may be better suited for achieving a more transient CRISPR effector expression, which could reduce off-target effects. Prolonged expression and off-target editing could also potentially be managed by designing CRISPR enzymes with improved specificity or that can self-inactivate. Rigorous preclinical characterization is mandatory to measure gene-editing activity in on-target and off-target tissues, including the liver and germline tissues. For example, a detailed preclinical study of VERVE-101 (an LNP-based base editor) evaluated liver enzymes, off-target editing, and germline editing up to 476 days after administration in mice, and no evidence of germline editing was observed. However, the FDA requested additional data on delivery in off-target (i.e., non-liver) cells for this program.[29]

7.2 Immunogenicity

Immunogenicity is a critical consideration when using viral vectors for the *in vivo* delivery of CRISPR-Cas9-based artificial transcription factors (ATFs) for cancer therapy.

Viral Vector Delivery Challenges:

- Viral vectors, such as adenovirus and lentivirus, are frequently used as delivery systems due to their high efficiency.

- However, a major drawback of these viral vectors is their potential for carcinogenesis and immunogenicity, which can lead to side effects.
- The ideal *in vivo* delivery system for the dCas9/sgRNA complex should aim to cause low immunogenicity.
- Newer administration methods, like the use of metal, polymeric, or lipid nanoparticles via nanotechnology, have emerged as alternatives. These nanoparticle-based systems may help to decrease systemic toxicity and immune risks associated with the transfection of the CRISPR components.[30]

7.3 Cellular uptake & endosomal escape

The challenge of achieving efficient cellular uptake and endosomal escape represents a critical limitation for the therapeutic translation of the CRISPR-Cas system. Effective delivery of the CRISPR machinery remains a formidable challenge, especially because the large size of Cas proteins (e.g., ~4.2 kb coding sequence for SpCas9) often approaches or exceeds the packaging limits of many viral vectors. This necessitates using either split-Cas systems or smaller orthologs, which can compromise activity or specificity.

To address these constraints, nonviral modalities are being developed. These include lipid nanoparticles, gold nanoparticles, and polymeric carriers, which show promise for transient delivery of Cas ribonucleoprotein complexes. This transient delivery method reduces off-target and immunogenic problems. However, successfully achieving tissue-specific targeting, efficient cellular uptake, and subsequent endosomal escape continues to impede robust *in vivo* editing. In the context of solid tumors, these challenges are intensified by the dense extracellular matrix and heterogeneous vascularization, which can further obstruct nanoparticle penetration, thereby limiting the therapeutic approach. Advances in nanomedicine, such as lipid nanoparticle formulations optimized for endosomal escape, have already demonstrated efficient *in vivo* liver editing in preclinical models. [31]

7.4 Ethical, biosafety, regulatory issues

Safety and Biosafety: A major challenge is the relatively high off-target effect of the system in its current form, which prevents its safe, direct application in the human body. Off-target cleavage can lead to a heterogenic population of edited cells, including cells cut at the wrong site in the genome, which is a massive safety concern for therapeutic gene editing. The goal for an acceptable off-target cleavage rate is arguably 0% for safe human application.

Ethical and Regulatory Issues: The human embryo editing experiments, though performed on nonviable zygotes, generated controversy, and the high off-target cleavage and low efficiency led authors to conclude that the system needs significant improvement before it can be applied in a clinical setting. This also touches upon the ethical debate surrounding germline editing. The debate over intellectual property ownership has also initiated a fierce patent war among the front-runners in the technology's adaptation. The regulatory path for CRISPR-Cas9 appears to be following that of RNA interference (RNAi), suggesting a lengthy process for clinical translation if effective delivery technology is not fully established. [32]

8. FUTURE PERSPECTIVES

8.1 dCas9, base/prime editors

Future innovations are focused on enhancing the precision and versatility of existing Cas systems, particularly through nuclease-inactivated effectors and advanced editing modalities. The single-effector Cas proteins, such as Cas9 and Cas13, can be deactivated to form dead Cas (dCas) variants. These dCas proteins function as RNA-guided binding domains for the recruitment of effector modules to modulate, monitor, or modify target nucleic acids.

- Applications of dCas-fusion proteins include targeted gene repression using the Krüppel-associated box (KRAB) domain, transcriptional activation, and editing of the epigenome via fusion with epigenetic modifiers. Furthermore, dCas variants are increasingly utilized for dynamic imaging and spatial manipulation of genomic organization in living cells.
- The development of base and prime editors represents a significant leap towards correcting the majority of pathogenic single-nucleotide point mutations, which constitute over half of all known pathological variants. Base editors, created by fusing dCas9 or a Cas9 nickase with single-strand DNA deaminases, enable the targeted, high-precision conversion of C - G to T - A or A - T to G - C in genomic DNA without inducing a double-strand break (DSB). This DSB-free mechanism is inherently safer, reducing the risk of large deletions or translocations associated with conventional Cas9-mediated DSBs.
- Expanding upon this concept, prime editing offers even greater versatility, permitting precise insertions, deletions, and replacements of up to dozens of base pairs without relying on DSBs or donor DNA templates.
- RNA editing, mediated by the fusion of dCas13 to an adenine deaminase acting on RNA (ADAR), provides a transient and reversible therapeutic option. This system achieves A-to-I (read as G) conversion in the transcriptome. The reversibility and non-permanent modification of the genome further expand the therapeutic utility of Cas systems for non-genetic inflammatory disorders, where CasRx-mediated RNA editing has generally shown greater efficacy than genome DNA editing.[33,34,35]

8.2 Al-driven nanocarriers & nanorobotics

A critical barrier to realizing the full potential of CRISPR therapeutics is the efficient and safe *in vivo* delivery of the large Cas editor payload. Future breakthroughs will rely on integrating Cas systems with sophisticated nanotechnology, notably AI-driven nanocarriers and advanced biosensing. The development of novel synthetic vectors is driven by advancements in material science and nanotechnology, which aim to resolve the limitations of viral vectors, such as small cargo size and immunogenicity, and overcome the low target specificity and toxicity of early non-viral vectors.

- AI-driven computational models show great promise for forecasting implant outcomes and optimizing nanocarrier design. Machine learning algorithms, trained on

extensive datasets of material properties and patient-specific characteristics, can simulate implant performance and predict long-term stability. This capability aids in implant conception and personalized treatment planning, potentially reducing the need for time-consuming and costly preclinical animal testing.

- The next generation of nanocarriers will feature enhanced targeting mechanisms. Examples include poly(disulfide) nanocomplexes coated with biomimetic macrophage membranes to specifically direct delivery to liver inflammatory lesions, thereby avoiding unwanted editing in non-hepatic tissues. Another strategy involves multi-component assembly, such as lipid-encapsulated gold nanoparticles, to achieve thermo-triggered release of the CRISPR system in tumor tissues.
- Furthermore, the integration of real-time biosensing and smart implants will revolutionize patient monitoring and therapeutic feedback. These smart implants, equipped with continuous glucose monitors or battery-less wireless sensors for vascular metrics, enable real-time observation of the implant-tissue interface. This continuous data flow is vital for early problem detection and for refining both surgical methods and implant designs.[36,37,38]

8.3 Personalized CRISPR therapeutics

The convergence of CRISPR and personalized medicine is transforming the therapeutic landscape, aiming to tailor treatment to individual patient genetics and disease characteristics. Genetically Engineered Animal Models (GEAMs) are crucial for this transition, offering increased physiological relevance for preclinical validation of next-generation surgical devices and implants.

- GEAMs can precisely mimic specific human disease states, such as osteoporosis or cancer-related mutations, which allows researchers to assess implant performance in a clinically relevant context.
- Personalized medicine seeks to align healthcare individually with the patient, maximizing quality of life and treatment results. GEAMs can be used to describe new implant performance by defining material-dependent and scaffold-architecture-dependent bone growth phenomena.
- In cancer immunotherapy, CRISPR-Cas9 is pivotal for creating customized T-cell therapies. The technique facilitates multiplex genome engineering to construct universal chimeric antigen receptor (CAR) T-cells, which are "off-the-shelf" allogeneic agents. This involves simultaneously knocking out endogenous T-cell receptors (TCRs) and Human Leukocyte Antigen (HLA) components to prevent competition with transgenic receptors and immune rejection.

As the field advances, addressing safety concerns (off-target effects), optimizing delivery systems, and navigating ethical considerations remain central to realizing the full promise of personalized CRISPR-based therapeutics and improving patient outcomes in a tailored manner.[39,40]

9. CONCLUSION

The CRISPR–Cas system has undeniably revolutionized biomedical science, providing a way to edit genomes with unprecedented precision and flexibility. However, the realization of its full therapeutic potential faces a critical bottleneck the need for delivery systems that can transport these components to specific tissues safely and effectively.To solve this nanotechnology-based carriers including lipid nanoparticles, polymeric systems, inorganic particles, and extracellular vesicles are emerging as superior alternatives to viral vectors. These nanocarriers offer better biocompatibility and safer intracellular access, effectively overcoming major hurdles such as cargo instability and the difficulty of penetrating cell membranes. While CRISPR-based delivery has shown immense potential in treating cancer, genetic disorders, infectious diseases, and neurological conditions, the path forward is not without obstacles. Challenges such as off-target edits, potential immune reactions, and complex regulatory and ethical issues still need to be addressed. Moving past these limitations will require the engineering of more precise Cas variants and the design of highly optimized, controllable delivery platforms. Looking to the future, the integration of innovations like base and prime editors, dCas9 regulators, and AI-driven smart nanocarriers promises to reshape the clinical landscape. Together, these advancements suggest a future where CRISPR-enabled delivery becomes the cornerstone of personalized, precision medicine.

REFERENCE

1. Wilbie D, Walther J, Mastrobattista E. Delivery Aspects of CRISPR/Cas for in Vivo Genome Editing. Acc Chem Res. 2019;52(6):1555–64. doi:10.1021/acs.accounts.9b00106.
2. Huang J, Zhou Y, Li J, Lu A, Liang C. CRISPR/Cas systems: Delivery and application in gene therapy. Front Bioeng Biotechnol. 2022;10:942325. doi:10.3389/fbioe.2022.942325.
3. Liu X, Gao M, Bao J. Precisely Targeted Nanoparticles for CRISPR-Cas9 Delivery in Clinical Applications. Nanomaterials. 2025;15(7):540. doi:10.3390/nano15070540.
4. Yang Y, Xu J, Ge S, Lai L. CRISPR/Cas: Advances, Limitations, and Applications for Precision Cancer Research. Front Med. 2021;8:649896. doi:10.3389/fmed.2021.649896.
5. Golkar Z. CRISPR: a journey of gene-editing based medicine. *Genes Genomics.* 2020;42:1369–80. doi:10.1007/s13258-020-01002-x

6. Liu C, Zhang L, Liu H, Cheng K. Delivery Strategies of the CRISPR-Cas9 Gene-Editing System for Therapeutic Applications. *J Control Release.* 2017;266:17–26. doi:10.1016/j.jconrel.2017.09.012
7. Tian X, Gu T, Patel S, Bode AM, Lee M, Dong Z. CRISPR/Cas9 – An evolving biological tool kit for cancer biology and oncology. *npj Precis Oncol.* 2019;3:8. doi:10.1038/s41698-019-0080-7.
8. Shamsi A, Alrouji M, AlOmeir O, Tasqeruddin S, Dinislam K, Zuberi A. CRISPR-Cas9: bridging the gap between aging mechanisms and therapeutic advances in neurodegenerative disorders. *Front Cell Neurosci.* 2025;19:1681891. doi:10.3389/fncel.2025.1681891.
9. Albhar KG, Gomase VS, Kemkar KR, Linge SG, Wagh VS, Vijayalakshmi A, Potnis VV. Drug Delivery System: A Novel Approach to Formulation Development. *Int J Pharm Sci Rev Res.* 2023;79(2):218–26. doi:10.47583/ijpsrr.2023.v79i02.035. (
10. Duan L, Ouyang K, Xu X, Xu L, Wen C, Zhou X, Qin Z, Xu Z, Sun W, Liang Y. Nanoparticle Delivery of CRISPR/Cas9 for Genome Editing. *Front Genet.* 2021;12:673286. doi:10.3389/fgene.2021.673286.
11. Khawaja S, Ali RH, Ahmed I, Umair M. Gene Therapy in Rare Genetic Disorders: Current Progress and Future Perspectives. *Curr Genomics.* 2025;26(4):278–89. doi:10.2174/0113892029361490250310041259.
12. Amiri S, Adibzadeh S, Khazaei Monfared Y, Kaboli S, Arashkia A, Barkhordari F, Mahmoudian M, Kheirandish MH, Trotta F, Davami F. Efficient Delivery of CRISPR-Cas9 RNP Complexes with Cyclodextrin-Based Nanosponges for Enhanced Genome Editing: TILD-CRISPR Integration. *Int J Mol Sci.* 2025;26(21):10682. doi:10.3390/ijms262110682.
13. Yang Y, Xu J, Ge S, Lai L. CRISPR/Cas: Advances, Limitations, and Applications for Precision Cancer Research. *Front Med.* 2021;8:649896. doi:10.3389/fmed.2021.649896.
14. Sinclair F, Begum AA, Dai CC, Toth I, Moyle PM. Recent advances in the delivery and applications of nonviral CRISPR/Cas9 gene editing. *Drug Deliv Transl Res.* 2023;13:1500–19. doi:10.1007/s13346-023-01320-z.
15. Glass Z, Lee M, Li Y, Xu Q. Engineering the Delivery System for CRISPR-based Genome Editing. Advanced Drug Delivery Reviews. 2017;121:182–97. doi:10.1016/j.addr.2017.08.001.
16. Du Y, Liu Y, Hu J, Peng X, Liu Z. CRISPR/Cas9 systems: Delivery technologies and biomedical applications. Asian J Pharm Sci. 2023;18(5):100854. doi:10.1016/j.ajps.2023.100854.
17. Bhat AA, Nisar S, Mukherjee S, Saha N, Yarravarapu N, Lone SN, Masoodi T, Chauhan R, Maacha S, Bagga P, Dhawan P, Akil A AS, El-Rifai W, Uddin S, Reddy R, Singh M, Macha MA, Haris M. Integration of CRISPR/Cas9 with artificial intelligence for improved cancer therapeutics. J Transl Med. 2022;20(1):534. doi:10.1186/s12967-022-03765-1.
18. Prakash M, Cortez CD, Jayaraman A, Hsu SY, Huang YC, Yeh CY, Lee YL. Innovative gene engineering and drug delivery systems for dendritic cells in cancer immunotherapy. J Biomed Sci. 2025;32(1):95. doi:10.1186/s12929-025-01191-1.

19. Miri SM, Tafsiri E, Cho WCS, Ghaemi A. CRISPR-Cas, a robust gene-editing technology in the era of modern cancer immunotherapy. Cancer Cell Int. 2020;20(1):456. doi:10.1186/s12935-020-01546-8.
20. Peng W, Zhao Q, Chen J, Peng H, Jiang H. Gene therapy for disorders of sex development: current applications and future challenges. Front Genet. 2025;16:1661127. doi:10.3389/fgene.2025.1661127. Chavez M, Chen X, Finn PB, Qi LS. Advances in CRISPR therapeutics. Nat Rev Nephrol. 2023;19(1):9–22. doi:10.1038/s41581-022-00656-2.
21. Javaid D, Ganie SY, Hajam YA, Reshi MS. CRISPR/Cas9 system: a reliable and facile genome editing tool in modern biology. Mol Biol Rep. 2022;49(12):12133–50. doi:10.1007/s11033-022-07880-6.
22. Seraj ZI, Haque S. Remodelling of a bacterial immune system as the simple gene editing tool, Crispr-Cas, for food security and human health. J Bangladesh Acad Sci. 2021;45(2):131–45. doi:10.3329/jbas.v45i2.57208.
23. Kazemian P, Yu SY, Thomson SB, Birkenshaw A, Leavitt BR, Ross CJD. Lipid-Nanoparticle-Based Delivery of CRISPR/Cas9 Genome-Editing Components. Mol Pharmaceutics. 2022;19(5):1669–86. doi:10.1021/acs.molpharmaceut.1c00916.
24. Westarp P, Keller T, Brand J, Horvat S, Albrecht K, Beisel C, Groll J. Efficient encapsulation of CRISPR-Cas9 RNP in bioreducible nanogels and release in a cytosol-mimicking environment. Discover Nano. 2025;20(1):119. doi:10.1186/s11671-025-04316-5.
25. Baliou S, Adamaki M, Kyriakopoulos AM, Spandidos DA, Panayiotidis M, Christodoulou I, Zoumpourlis V. CRISPR therapeutic tools for complex genetic disorders and cancer (Review). Int J Oncol. 2018;53(2):443–68. doi:10.3892/ijo.2018.4434.
26. Leung AKK, Tam YYC, Cullis PR. Lipid Nanoparticles for Short Interfering RNA Delivery. Adv Genet. 2014;88:71–105. doi:10.1016/B978-0-12-800148-6.00004-3.
27. Peng C, Zheng M, Ding L, Chen X, Wang X, Feng X, Wang J, Xu J. Accurate Detection and Evaluation of the Gene-Editing Frequency in Plants Using Droplet Digital PCR. Front Plant Sci. 2020;11:610790. doi:10.3389/fpls.2020.610790.
28. Madigan V, Zhang F, Dahlman JE. Drug delivery systems for CRISPR-based genome editors. Nat Rev Drug Discov. 2023;22(11):875–94. doi:10.1038/s41573-023-00762-x.
29. Martinez-Escobar A, Luna-Callejas B, Ramón-Gallegos E. CRISPR-Cas9-Based Artificial Transcription Factors to Improve Efficacy of Cancer Treatment With Drug Repurposing: Proposal for Future Research. Front Oncol. 2021;10:604948. doi:10.3389/fonc.2020.604948.
30. Park SJ, Lee GE, Cho SM, Choi EH. Recent applications, future perspectives, and limitations of the CRISPR-Cas system. Mol Ther Nucleic Acids. 2025;36:102634. doi:10.1016/j.omtn.2025.102634.
31. Oude Blenke E, Evers MJW, Mastrobattista E, van der Oost J. CRISPR-Cas9 gene editing: Delivery aspects and therapeutic potential. J Control Release. 2016;244:139–48. doi:10.1016/j.jconrel.2016.08.002.

32. Miri SM, Tafsiri E, Cho WCS, Ghaemi A. CRISPR-Cas, a robust gene-editing technology in the era of modern cancer immunotherapy. Cancer Cell Int. 2020;20(1):456. doi:10.1186/s12935-020-01546-8.
33. Sari R, Syafiq M, Anwari M. Recent progress in nanocarriers for CRISPR-Cas9 genome editing delivery. J Drug Target. 2023;31(4):379–96. doi:10.1080/1061186X.2023.2191599.
34. Kim J, Ma F, Wang Y, Chen T, Yu W, Geng J, Fan X, Zhang D. Current advances and challenges in non-viral delivery systems for therapeutic CRISPR/Cas9. View. 2023;4(1):20220037. doi:10.1002/vie.20220037.
35. Gong Z, Huang Z, Li X, Liu Z. Progress and prospects of non-viral vectors for CRISPR-Cas gene editing system delivery. Eur J Pharm Biopharm. 2023;184:10–22. doi:10.1016/j.ejpb.2023.01.002
36. Alshehri AA, Al-Aboody MS, Alqahtani FM, Al-Garni MS, Alsuwailem AE, Al-Dossari AM. Applications of CRISPR/Cas9 in cancer immunotherapy and associated complications. Saudi J Biol Sci. 2023;30(3):103562. doi:10.1016/j.sjbs.2023.103562.
37. Alshehri AA, Al-Aboody MS, Alqahtani FM, Al-Garni MS, Alsuwailem AE, Al-Dossari AM. Applications of CRISPR/Cas9 in cancer immunotherapy and associated complications. Saudi J Biol Sci. 2023;30(3):103562. doi:10.1016/j.sjbs.2023.103562.
38. Wang S, Chen S, Jin X, Deng H, Chen D, Fan F, Wu S, Zhang H, Wu L, Zhang P, Li X. Recent advances in CRISPR/Cas9-mediated cancer immunotherapy. J Hematol Oncol. 2021;14(1):63. doi:10.1186/s13045-021-01072-8.
39. Li C, Liu B, He L, Li G, Wu S. Recent developments in CRISPR/Cas9-mediated in vivo therapy. Acta Biochim Biophys Sin. 2020;52(10):1136–45. doi:10.1093/abbs/gmaa105.
40. Wang L, Lu Z, Li Y, Chen L, Yin H, Jiang Z. Delivery of CRISPR/Cas systems for gene-editing and gene regulation applications. Acta Pharm Sin B. 2022;12(5):2245–66. doi:10.1016/j.apsb.2021.11.010.