



NANOTECHNOLOGY-ENABLED NON-VIRAL DELIVERY SYSTEMS FOR CRISPR/CAS9 GENE THERAPY: CURRENT PROGRESS AND TRANSLATIONAL CHALLENGES

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ABSTRACT

CRISPR/Cas9 has become a versatile platform for programmable genome editing and has generated substantial interest as a therapeutic modality for inherited and acquired diseases. However, clinical translation depends not only on editing specificity but also on safe and efficient delivery of the Cas9 nuclease and guide RNA to the intended cells. Viral vectors can provide high transduction efficiency, but concerns related to immunogenicity, payload constraints, manufacturing complexity and, for some vectors, genomic integration have encouraged development of non-viral alternatives. This review examines nanotechnology-enabled non-viral delivery strategies for CRISPR/Cas9, with emphasis on plasmid DNA, messenger RNA and ribonucleoprotein cargo formats. Major delivery barriers – including extracellular degradation, protein-corona formation, limited tissue penetration, cellular uptake, endosomal entrapment, intracellular trafficking and nuclear access – are discussed together with strategies for overcoming them. Lipid nanoparticles, polymeric nanoparticles, lipid-polymer hybrids, dendrimers, inorganic nanoparticles and emerging vesicular or virus-like systems are compared in terms of cargo compatibility, targeting, endosomal escape, safety and translational potential. Recent advances in liver-directed in vivo editing, non-liver targeting, stimuli-responsive delivery and clinical translation are highlighted. Particular attention is given to the need for tissue-selective delivery, transient editor exposure, reproducible manufacturing and long-term safety monitoring. Overall, rationally engineered, biodegradable and multifunctional nanocarriers represent an important route toward safer and more effective CRISPR/Cas9 therapeutics.

KEYWORDS: CRISPR/Cas9; nanotechnology; non-viral delivery; lipid nanoparticles; gene therapy; genome editing.

1. INTRODUCTION

CRISPR/Cas9 has moved from a bacterial adaptive immune mechanism to one of the most widely used programmable genome-editing platforms. The therapeutic concept is attractive because a guide RNA can direct Cas9 to a selected genomic sequence, where DNA cleavage is followed by endogenous repair or donor-directed correction. Nevertheless, the therapeutic problem is substantially broader than target recognition: an active editor must reach the correct tissue and cell, remain functionally intact, enter the appropriate intracellular compartment and act for a sufficiently short period to limit unwanted editing. Accordingly, delivery is now recognized as a central determinant of the safety and efficacy of genome-editing medicines.^[1,2,3,4]

Nanotechnology is the science, technology, and engineering of designed and fabricated materials or structures that are measured in units of nanometers (1–100 nm).^[1,2,3,4]

A non-viral delivery system is a strategy or vehicle by which therapeutic agents like DNA, RNA, protein, and CRISPR/Cas9 are delivered to a target cell without the use of a virus.^[1,2,3,4]

CRISPR is the guide system and guide RNA used to find the specific DNA sequence to be cut. Cas9 is an enzyme that acts as molecular scissors that can cut DNA near or at the specific location. CRISPR/Cas9 gene therapy is a new approach to disease treatment that involves the use of a CRISPR/Cas9 gene-editing technique for altering a particular DNA region in a targeted manner.^[1,2,3,4]

Background of gene therapy

The CRISPR system was discovered thanks to some strange short repeats found in bacterial genomes. The presence of these repeating sequences is used by CRISPR/Cas systems; they flank protein-encoding genes, which are interspersed with spacers that were actually foreign DNA. We learned that the CRISPR-Cas systems have acquired immunity and are thus immune and function via cleaving foreign DNA molecules.^[1,2,3]

The mechanism by which the bacteria become aware is using unique spaced DNA repeats found within CRISPR sequences, as opposed to the typical DNA structure to recognise, for example, viruses as foreign.^[1,2,3]

In 2012, it was shown that CRISPR-Cas can cut DNA at any desired sequence, since designing a suitable RNA molecule targeting the DNA sequence of interest is straightforward. CRISPR-Cas is composed of only two genes, Cas and RNA, so it is feasible for use in genetic engineering applications. In fact, it has been employed in more than a thousand laboratories worldwide and is extremely accurate for genome editing. Modified Cas9 versions have been generated, as are high-fidelity nucleases, base editors, prime editors and various CRISPR-associated proteins.^[1,2,3]

CRISPR/Cas9 as a genome-editing technology

CRISPR/Cas9 has brought to life a new era for genome engineering due to its programmable and highly efficient nature. The development of this technology has enabled the creation of gene knockouts, targeted genome alterations, and cellular engineering. Despite the limitations and ethical concerns that CRISPR technology faces today, the continued evolution of this innovation has the potential to overcome many challenges, including off-target effects, limitations of delivery methods, safety, and more. Specifically, progress in the engineering of the Cas protein, guide RNA, base editing, prime editing, and delivery methods will contribute to the advancement of CRISPR genome-editing technology.^[1,2,3]

Importance of efficient delivery

Efficient delivery mechanisms are a critical component of any successful gene therapy. The development of CRISPR/Cas9 and other genome-editing technologies is unlikely to surpass its therapeutic potential without safe and effective means of delivering its molecular constituents to the target cells. A growing number of approaches to overcome this challenge are being explored, including viral-vector engineering, lipid nanoparticles, polymeric systems, targeted delivery and transient genome editing. Progress in these areas will undoubtedly facilitate the development of more effective and safer genetic therapies.^[1,2,3]

Need for non-viral delivery systems

The development of non-viral delivery platforms is driven by the effort to overcome key shortcomings of the viral vectors while achieving efficient intracellular delivery. Their potential for very low immunogenic response, the possibility to pack a wide range of payload capacity and tune the number of transgenes delivered, capability of transient expression, easily modifiable physical and chemical properties, and easier manufacturing process make these carriers desirable for both modern gene therapy applications and for gene editing applications of the CRISPR/Cas9 system. With the on-going progress in lipid nanoparticles, polymeric carriers, targetable nanocarriers and related delivery technologies, non-viral systems may have better delivery efficiency and specificity. It is thus envisioned that by further development of safe and efficient tissue-specific non-viral delivery carriers and manufacturing, gene editing applications may be successfully incorporated into clinical practice.^[4,5,7,11]

Advantages of Non-Viral Delivery Systems

Non-viral delivery systems are a good alternative to viral ones for delivering therapeutic genes since they lack many of the limitations of the latter. For instance, non-viral delivery systems eliminate the possibility of immunological response against the vector and allow flexible packaging of various types and sizes of genetic material. The expression of the gene is transient, which is appropriate for certain applications. Moreover, their physicochemical properties can be easily engineered to

target specific cells or tissues, allow multiple administrations, are scalable, and can deliver more than one type of CRISPR/Cas9 component. However, their low transfection efficiency, poor endosomal escape, inability to target specific tissues and organs, and safety in the case of repeated administration remain significant drawbacks that need to be addressed to make non-viral delivery systems more clinically relevant.^[4,5,7,11]

2. CRISPR/CAS9 GENE EDITING SYSTEM

CRISPR/Cas9 gene editing technology has made significant contributions to the development of molecular genetics by providing new opportunities for targeted genome modification. This technology has had a major impact on the field of molecular genetics due to its versatility, programmability and relative accessibility. Thanks to this technology, it has become possible to obtain gene knockouts, make targeted changes to specific DNA sites, and edit cells for therapy. Despite this, difficulties in the way of achieving perfect gene mutation are numerous. The development of methods for the implementation of gene editing and solving some of the accompanying difficulties will allow taking this technology to a new level.^[1,2,3]

Components of the CRISPR/Cas9 system

The major components of the CRISPR/Cas9 gene-editing system are:

Cas9 Protein

The protein Cas9 is a prokaryotic enzyme belonging to the class of RNA-guided DNA endonucleases. This enzyme is capable of being programmed in order to cut DNA at desired locations. The protein consists of two major domains: the REC and NUC lobes. These lobes, along with the HNH, RuvC, and PAM-interacting domains, are responsible for the specific binding of guide RNA, targeting of DNA for cutting by means of the PAM sequence, unwinding of DNA strands and cleavage of DNA strands. Due to the possibility of modifying the Cas9 protein, scientists were able to create nickases, dCas9-based gene regulation systems, and altered Cas9 proteins with improved targeting characteristics. Although there are some challenges associated with the use of this protein, such as off-target effects, the limitation of available PAM sequences, and the necessity of protein delivery, these issues will be resolved due to continuous improvements to the CRISPR/Cas9 genome-editing system.^[1,2,3]

Guide RNA (gRNA)

Guide RNA is the critical determinant of the precision and efficiency of the CRISPR/Cas9 genome editing platform. By virtue of sequence-specific target DNA recognition, guide RNA directs the position of Cas9 nuclease recruitment and hence dictates the site of genome modification. The development of engineered sgRNAs, improved computational design approaches, chemical modifications and machine-learning-based activity prediction has expanded the potential of guide RNA technology. However, there are still challenges

related to off-target effects, activity variability, PAM-dependence, RNA stability and delivery. Thus, there is a need for the optimization of gRNA sequences, structure and chemical features to enable more precise and efficient genome editing, especially in the context of therapeutic applications.^[1,2,3]

Target DNA

Target DNA sequences are necessary for the CRISPR/Cas9 genome-editing system as they determine where in the genome edits will be made. The recognition of target DNA sequences is mediated through PAM detection, local DNA unwinding to access the target sequence, and hybridisation of the gRNA with the target DNA sequence to generate an R-loop. When target DNA has been recognized, the Cas9 nuclease domains are activated, and double-strand DNA breaks can be introduced at the desired genomic location; this is followed by an internal DNA repair mechanism. The success and specificity are heavily reliant upon the availability of a protospacer adjacent motif (PAM), complementary base-pairing between guide RNA (gRNA) and target DNA, the specific sequence of the target DNA, the context in the genome, and chromatin availability. This makes highly specific choice and validation of the target DNA sequences critical to prevent off-target events (particularly important for therapies) and maximize accuracy.^[1,2,3]

PAM (Protospacer Adjacent Motif)

PAM stands for the protospacer adjacent motif. The PAM is a very important aspect of CRISPR/Cas genome engineering. It is directly recognized by the Cas protein and thus is one of the key sequences to target the DNA. For the most common system, SpCas9, the PAM is 5'-NGG-3', recognition of which initiates DNA unwinding, guide RNA-DNA hybridization, and activation of the Cas9 nuclease.^[1,2,3]

It is absolutely necessary for targeting DNA and genome editing to target this specific PAM, which is a further limitation in terms of the genomic loci to be targeted. The identification and characterization of numerous Cas proteins and engineered PAM-expanded versions of Cas9 (Cas9-NG, xCas9, SpG and SpRY) open a wide range of applications of CRISPR systems. Yet, targeting specificity is sacrificed when expanding targeting ability, and each new Cas version must be checked individually. Thus, the study of PAMs plays a very important role in deciding the ability and utility of the various genome engineering systems.^[1,2,3]

Repair Template

The repair template is an essential element of specific genome editing by means of CRISPR/Cas9, especially when one intends to obtain a particular modification rather than just induce gene inactivation. The given template provides the necessary sequence information along with homology to allow for the desired correction, insertion, or replacement via HDR. Both single- and

double-stranded DNA can be used as a donor sequence; however, their respective structure, homology regions, delivery mechanism, and repair pathways utilized by cells upon introduction all play a critical role in dictating the outcome of editing. While HDR is generally accepted to be less efficient than NHEJ, improvements in donor optimization and delivery techniques will enable achieving higher levels of precision and therapeutic efficacy for gene correction using CRISPR/Cas9.^[1,2,3]

Mechanism of CRISPR/Cas9-Mediated Genome Editing

The mechanism of CRISPR/Cas9-mediated genome editing includes multiple sequential molecular events that start from the formation of the Cas9–gRNA complex and PAM recognition, the binding with target DNA, the opening of the DNA double strand to create an R-loop structure, the activation of the Cas9 nuclease and the cutting of both strands to form a double-strand break (DSB). Cellular DNA repair mechanisms, mainly NHEJ and HDR, are then engaged to repair the DSB to induce gene-disruptive indel formation or accurate genetic modification. The ease of use and programmability of this system have made Cas9–gRNA genome editing one of the most impactful gene-editing platforms used in modern biotechnology and biomedicine. However, improvement of target specificity, delivery, DNA repair outcome and long-term safety are still required to achieve its full potential as a therapeutic platform.^[1,2,3]

Target Recognition and gRNA Binding

Target recognition and gRNA binding are complex processes in CRISPR/Cas9 genome editing. First, a Cas9–gRNA binary complex is formed, followed by DNA unwinding and guide–target pairing, leading to R-loop formation and Cas9 conformational activation. The seed region of the gRNA is critical for target recognition, while gradual R-loop formation appears to drive the structural changes in Cas9 that are required for nuclease activity. PAM recognition and extensive guide–target complementarity are critical for target-specific DNA cleavage. Nevertheless, these mechanisms can sometimes allow for off-target interactions. This knowledge can be used to develop improved genome editing technologies based on CRISPR/Cas9.^[1,2,3]

Cas9-Mediated DNA Cleavage

The Cas9 protein has a double nuclease activity composed of two different nuclease domains: HNH and RuvC. The first domain, the HNH nuclease, cleaves the complementary strand. The second, the RuvC nuclease, cleaves the non-complementary strand. For the wild-type SpCas9, this cut is made three bases before the PAM sequence, producing a DNA double-strand break with blunt ends. A targeted DSB activates the cell's DNA repair systems, which provide different final outcomes.^[1,2,3]

DNA Repair and Genome Editing Outcomes

DNA repair determines the outcomes of the process of genome editing by means of CRISPR/Cas9. After the incision made by Cas9, the cell can utilize a variety of repair pathways, including NHEJ, MMEJ, HDR, or other mechanisms, leading to different repair outcomes. For instance, while NHEJ usually causes insertions and deletions at the site of the break and, therefore, is used for gene knockout, HDR can repair the site using a provided template. The choice of repair pathway depends on a multitude of factors, including cell cycle, targeted location, chromatin state, structure of the DNA ends, availability of repair templates, and cell type. Therefore, understanding DNA repair mechanisms is a crucial step in upholding the accuracy of genome editing and, thus, its results. Recent studies are working on controlling the outcome of repair at the level of individual cells and developing alternative methods of genome editing capable of reducing unwanted mutations. The study of DNA repair pathways lays the foundation for the precision of genome editing and, consequently, gene therapy.^[1,2,3]

Overall Mechanism

The entire process can be depicted as flowing sequentially, encompassing sgRNA selection, assembly of the sgRNA–Cas9 complex, binding of the sgRNA–Cas9 complex to a specific DNA sequence, and cutting of the DNA. Specificity comes from the specificity of the guide RNA and the specificity for a PAM sequence. The cellular DNA repair enzymes are responsible for determining the type of repair of the cut in the DNA molecule. For example, DNA damage can be repaired via the HDR and NHEJ pathways, but the outcome of homology-directed repair at a given site depends solely on the cell's HDR capacity.^[1,2,3]

DNA repair pathways: NHEJ and HDR

NHEJ and HDR are the two main DNA repair mechanisms, which dictate the outcome of editing induced by the CRISPR/Cas9 system. NHEJ is efficient, active during all stages of the cell cycle, and tends to induce indels within the genome, which makes it effective for gene knockout. HDR is a more refined, template-directed system, which can induce a specific sequence correction and/or insertion, though its efficiency is lower and HDR activity is mostly limited to dividing cells. The relative activity between these two pathways in response to editing is a key mediator in achieving successful and accurate editing, and determines its potential utility for therapy. Future investigation will involve manipulating these two repair pathways, optimising donor and delivery systems, and utilizing other editing methods in lieu of conventional HDR, all of which will allow for precise and reliable editing beneficial for future therapeutic interventions.^[7,10]

Non-Homologous End Joining (NHEJ)

NHEJ is an important DNA repair pathway that dramatically affects CRISPR/Cas9-induced editing

events in the genome. As NHEJ can rapidly recognize, process and ligate Cas9-induced DSBs, facilitating a wide range of gene disruption events, it has been most widely used for knockout applications. It mainly utilizes Ku70/Ku80, DNA-PKcs, DNA-end processing factors, and the XRCC4-DNA ligase IV complex. However, NHEJ is an error-prone pathway which may produce a mixed assortment of insertions or deletions that cannot be applied for precise gene modification.^[7,10] Hence, elucidation of the molecular mechanisms controlling the balance of NHEJ against other mechanisms (such as HDR or alternative end-joining pathways) will improve CRISPR/Cas9 gene editing process efficiency, specificity and predictability.^[7,10]

Homology-Directed Repair (HDR)

Homology-directed repair is considered one of the critical endogenous DNA repair pathways that allow highly precise changes and modifications of the genome after CRISPR/Cas9-induced double-strand DNA breaks. Through the use of a homologous donor template, HDR can be harnessed to perform targeted and precise nucleotide substitutions, including correction of disease-associated mutations as well as targeted insertions of genes, reporters, and tags. Its mechanism involves DNA-end resection, RAD51-mediated homology search, strand invasion, DNA synthesis, and resolution of repair products.^[7,10]

The key limitation of HDR is that it is typically less efficient than non-homologous end-joining. Furthermore, HDR proficiency is dictated by cell cycle state, availability of a donor template for HDR-mediated repair, cellular DNA repair capacity, and the efficiency of delivery of HDR components, among other factors. These limitations necessitate continued research into optimizing HDR for therapeutic applications in a variety of cell types, including post-mitotic cells. In summary, HDR represents one of the critical repair mechanisms that can be leveraged for targeted genome modifications and, thus, holds promise for advancing the field of genome engineering.^[7,10]

Regulation of the NHEJ–HDR Pathway Choice

NHEJ and HDR are competing pathways that act on a CRISPR/Cas9-induced double-strand break and are rigorously controlled through several mechanisms: DNA end protection, DNA end resection, cell cycle control, chromatin signalling, and orchestration by multiple DNA repair proteins. Ku70/Ku80 and 53BP1-related pathways are involved in DNA end protection and favor NHEJ, whereas MRN, CtIP, BRCA1 and RAD51-based pathways provide resection and favor HDR; the mutual repression exerted by 53BP1 and BRCA1 on each other is the main hub of pathway selection. Improving the precision and predictability of CRISPR/Cas9-mediated genomic editing depends upon an efficient understanding of repair pathway selection; NHEJ is desired when only efficient knockout is wanted, while accurate gene correction/knock-in requires favoring HDR. For future

development, especially with non-viral delivery, efficient and safe gene editing is anticipated to be achieved through combined optimized delivery and regulation of endogenous repair pathways.^[7,10]

NHEJ and HDR in CRISPR/Cas9 Genome Editing

The main factor influencing the outcome of an editing event is which pathway is used to repair the DSB generated by the Cas9 nuclease. If the error-prone NHEJ pathway dominates, it inserts or deletes a few nucleotides at the break, which can result in a loss-of-function gene; this outcome can be exploited to produce gene knockouts. Conversely, if HDR is used to repair the break, it results in targeted insertion of the edited sequence into the genome.^[7,10]

The proportional involvement of both pathways can be increased or decreased depending on experimental variables such as the design of the donor template, cell culture conditions, delivery mechanisms, or expression modulation of the factors responsible for these pathways. It is therefore of high interest to the genome editing field to enable shifting the repair pathway towards HDR when necessary.^[7,10]

3. Types of CRISPR/Cas9 Therapeutic Cargo: Plasmid DNA, mRNA and RNP

A critical but not yet adequately resolved issue of therapy based upon CRISPR/Cas9 genome editing systems lies not solely in the design of the guide RNA and Cas9 nuclease, but also in their delivery to the target cell. Plasmid DNA (pDNA), messenger RNA and ribonucleoprotein particles (RNP complexes) have all been thoroughly explored as possible CRISPR/Cas9 delivery vehicles. They are distinct in structure, in vivo kinetics, Cas9 persistence, delivery strategy, potency and safety. Hence, developing a delivery strategy is absolutely fundamental to the therapeutic application of CRISPR/Cas9.^[4,7,8,11]

Plasmid DNA

Plasmid DNA is a highly efficient and adaptable non-viral DNA platform that supports CRISPR/Cas9 genome editing, as a single plasmid can encode Cas9, gRNAs, and a homology-directed repair (HDR) template (when needed) to mediate gene disruption or gene editing with high precision. In addition, plasmid vectors are readily available and facile to construct, optimize, store and scale, and are therefore widely applied. However, the large physical size, inefficient cellular uptake of naked plasmid, mandatory passage through the nucleus and delay of protein expression, inevitable elicitation of innate immune responses, and potential prolonged Cas9 activity raise important hurdles to their widespread use, thus encouraging ongoing research into ways of reducing plasmid size, enhancing DNA quality, modulating expression cassettes, and developing effective non-viral carriers, including liposomes, polymeric carriers and biomaterial-based delivery technologies. Future progress towards safer, more efficient and scalable CRISPR/Cas9

genome-editing platforms will undoubtedly greatly benefit from improved plasmid vectors coupled with advanced non-viral delivery strategies.^[4,7,8,11]

Messenger RNA (mRNA)

Messenger RNA stands out as one of the most promising non-viral delivery vectors for the delivery of CRISPR/Cas9 for genome editing. The key strengths that underpin this strategy are high speed of cytoplasmic translation, transient nature of Cas9 activity, exclusion from the nuclear transcriptional cycle, independence from the delivery of template DNA, and flexibility in molecular engineering. Thus, mRNA holds a competitive edge over its counterparts for therapeutic applications when transient and temporally constrained nuclease activity is desired. However, inherent RNA instability and immunogenicity, inefficient cellular uptake, limited endosomal escape and lack of tissue specificity are important hurdles being addressed in part through modifications in mRNA engineering, modified nucleosides, lipid nanoparticles, and directed delivery vehicles.^[4,7,8,11]

CRISPR/Cas9 Ribonucleoprotein (RNP)

CRISPR/Cas9 RNP is one of the simplest, most controllable non-viral genome engineering systems. Because the protein complex of Cas9 and sgRNA is already assembled, it skips the two-step process (transcription and translation) of traditional plasmid-based Cas9 and sgRNA expression systems; hence, gene editing is faster and results in a shorter duration of intracellular nuclease exposure. This transient “hit and run” process could minimize prolonged Cas9 exposure time, potentially leading to decreased off-target cutting, and eliminates the need to deliver the gene for Cas9 expression.^[4,7,8,11]

The major drawback of RNP delivery technology is the challenge of delivering the bulky protein/RNA complex across intracellular and extracellular membranes. Many delivery platforms have been developed, such as electroporation, lipid nanocarriers, polymers, nanogels, cell-penetrating peptides and extracellular vesicles. Further refinement of these delivery methods, along with improved Cas9 engineering and smarter sgRNAs, would make RNP the optimal technology for ex vivo applications and eventually a viable delivery system for in vivo applications. CRISPR/Cas9 RNP is a platform with fast kinetics and minimal cell disruption or damage at the time of editing, and it also minimizes the usage of the Cas9 gene, which is beneficial for safety when compared to other systems.^[4,7,8,11]

Therapeutic Considerations

The choice of therapeutic CRISPR/Cas9 cargo depends on several factors, including the cell type and route of administration, the level and duration of Cas9 protein expression needed to induce the desired modifications, the efficiency of editing, the method of delivery, immunogenicity and safety. Plasmid DNA appears to be

a good choice if long-term expression is required, while mRNA and RNPs are more appropriate when short-term Cas9 expression is sufficient to obtain efficient editing.^[4,7,8,11]

When designing a genome editing therapy, it is often desirable to reduce the persistence of active Cas9 to minimize potential off-target effects. This consideration has stimulated the development and preclinical evaluation of various transient expression systems, among which are mRNA and RNPs. RNP delivery appears to be optimal in cases of an acute need for genome editing, while mRNA is a versatile tool that can be used in research and therapy when delivery of protein molecular complexes is problematic.^[4,7,8,11]

Therapeutic potential of CRISPR/Cas9

Genome editing with CRISPR/Cas9 is a novel technology that holds great promise for the treatment of genetically inherited and acquired diseases. The technology allows specific editing at a targeted locus and, through editing the genome, can correct a faulty gene, disable it, activate or inactivate it, modulate expression of certain genes, and even enable gene insertion. The accuracy of gene editing is preferable to previous methods of gene therapy at a targeted genomic locus. In the following sections, the application of CRISPR/Cas9 technology to treat various kinds of genetic diseases, cancer, infection, and other diseases induced by gene dysfunction is overviewed.^[9,10,11,15]

CRISPR/Cas9 for Genetic Disorders

The genetic correction of monogenic diseases is one of the greatest clinical implications of the CRISPR/Cas9 system. In addition, repair of mutated regions should allow recovery of gene function from the mutated allele. Sickle cell anemia, β -thalassemia, cystic fibrosis, Duchenne muscular dystrophy, and certain types of inherited retinopathy have all been presented as potential target diseases. With regard to blood disorders like sickle cell disease and β -thalassemia, CRISPR/Cas9 could be directed to drive iPSC differentiation into hematopoietic cells and correct regulatory regions to re-activate genes responsible for fetal hemoglobin, which complements adult hemoglobin gene(s).^[9,10,11,15]

Regarding a blood genetic disorder strategy with CRISPR/Cas9, patient hematopoietic stem cells are removed, gene-corrected in vitro, and then returned to the patient. The goal of such a strategy is replacement of deficient hematopoietic cells with normal blood cells derived from gene-corrected iPSC.^[9,10,11,15]

With respect to the use of CRISPR/Cas9 to combat Duchenne muscular dystrophy, some investigators suggest that deletion of a mutated segment (which causes loss of protein function) may allow correction of the reading frame. Hence, the CRISPR/Cas9 system can be utilized to target numerous difficult diseases.^[9,10,11,15]

Application in Cancer Therapy

Cancer is another area where CRISPR-based therapeutics has great scope. Apart from targeting the harmful germline mutation that causes cancer, CRISPR/Cas9 therapeutics also offer the option to edit genes associated with cancer so they behave properly in terms of tumour initiation and growth, regulation of the immune system, and resistance to treatment. In the meantime, editing the genome of immune cells could be a good way to target cancer by modifying immune cells to kill the tumour.^[9,10,11,15]

CRISPR/Cas9 could help in identifying targets. For example, CRISPR/Cas9 can be employed to edit gene(s) in the immune system, upregulating or editing gene(s) responsible for the anti-tumour response of the immune system and knocking out genes that suppress immune response. CRISPR/Cas9 allows development of immunotherapies by targeting a cancer's genes or editing the T cell genome so that a cancer can be specifically recognised and destroyed. Besides, this technique allows functional screens to find which genes play a role in proliferation, survivability, resistance to therapies, and other functions of cancer cells. Furthermore, this technique can also help identify which genes contribute to tumour immune evasion, which can facilitate the effect of immunotherapy in treating cancer.^[9,10,11,15]

Nevertheless, several issues must be dealt with when applying these techniques in the clinic, such as the heterogeneity of solid tumours, targeting efficiency, delivery through tumour tissue, unwanted off-target modification, and immune response to the tools/vectors that deliver CRISPR/Cas9 to the cell. Overall, a CRISPR approach to cancer therapeutics, despite its amazing prospects, is not without pitfalls and must be applied with utmost care.^[9,10,11,15]

Treatment of Infectious Diseases

CRISPR/Cas9 therapy can be used for treating infectious diseases since it allows targeting of the genetic material of pathogens as well as host cell genes and regulatory elements. The nucleic acid of various viruses can be found and destroyed using this method so that they cannot survive or replicate in a person's body.^[9,10,11,15]

The use of this technology has already been studied and tested on various viruses, including HIV, hepatitis B, and herpesviruses. Guide RNAs enable the Cas9 enzyme to locate the specific nucleic acid sequences of pathogens, which it then cuts and deactivates. Moreover, with the help of CRISPR, it is possible to study host genes and their role in the process of infection, as well as the effect of the virus on them.^[9,10,11,15]

Even though the technology has enormous potential, there are some challenges to using it to fight infections. First, it can be difficult to target all cells invaded by pathogens, and there is a risk that some will continue to survive and infect other cells. Moreover, viruses can

exist in different forms, making it hard to develop a single guide RNA, as well as raising the possibility of resistance developing against it.^[9,10,11,15]

CRISPR/Cas9 in Neurological and Neurodegenerative Disorders

Targeting and modification of disease-causing genes by CRISPR/Cas9 has generated hope that it may offer promising avenues to treat many neurological and neurodegenerative diseases, by correcting disease-causing mutations, suppressing disease-causing gene products, or modifying genes involved in disease progression. For many neurodegenerative diseases, genome-editing therapies have already been tested in disease models, such as Huntington's disease and ALS, among others. However, the clinical delivery of such genome editors to specific cell types, across a restricted target neuronal population in postmitotic CNS neurons, is of great interest due to the limited plasticity and access within the CNS. Therefore, development of strategies for targeted neuronal delivery of genome editing systems needs to be addressed.^[9,10,11,15]

CRISPR/Cas9 for Cardiovascular and Metabolic Disorders

In addition, CRISPR/Cas9 could help fight cardiovascular and metabolic disease by targeting relevant metabolic pathway genes, such as those involved in cholesterol metabolism and glucose homeostasis, and genes that dictate a patient's predisposition toward these diseases. Modification of genes responsible for unbalanced cholesterol metabolism, for instance, may help lower LDL cholesterol levels and thereby reduce the risk of cardiovascular disease development.^[9,10,11,15]

The treatment of inborn metabolic diseases due to enzyme or transcription factor malfunction is a main research goal in the field. Although these challenging cardiovascular and metabolic diseases typically have multifactorial aetiology, this strategy will need to be cautiously engineered.^[9,10,11,15]

4. BARRIERS TO CRISPR/CAS9 DELIVERY

Efficient and effective delivery of CRISPR/Cas9 system components to target cells and tissues is crucial for CRISPR-based therapies. However, although the CRISPR/Cas9 system shows promising potential to effectively treat genetic disorders, cancers, viral diseases, and various other ailments, it is still confronted with a major hurdle regarding delivery. Indeed, whereas small-molecule therapeutics entail delivery of a single, relatively simple entity, delivery of CRISPR/Cas9 components is a far more complicated scenario, because there are few vehicles suitable for molecules of this size and diversity that can prevent untimely degradation and ensure that they reach target tissues and cells. Thus, a well-designed delivery system that targets particular tissues or cells while minimising side effects remains the

most critical obstacle in the development of CRISPR-based therapeutics.^[4,5,7,11]

Extracellular Barriers to CRISPR/Cas9 Delivery

Extracellular barriers are the first challenge for CRISPR/Cas systems after introduction into the body. At the moment of delivery, components should resist degradation, remain in a form suitable for cellular uptake, and accumulate in sufficient amounts at the site of action. This is particularly relevant for plasmid DNA, mRNA, and Cas9-gRNA RNP as promising CRISPR/Cas9 delivery vehicles, all of which have to overcome multiple physiological barriers to gain access to target cells.^[4,5,7,11]

Degradation by Nucleases and Proteases

Unencapsulated CRISPR components based on nucleic acid (DNA or mRNA) are subject to degradation by extracellular nucleases in blood and interstitial fluid, whereby mRNA molecules are degraded by RNases and plasmid DNA by DNases. Moreover, the Cas9 protein in an RNP complex may be subject to proteases or may undergo a conformational change in vivo. Both effects have an impact on the amount of functional CRISPR components that penetrate into target cells and consequently limit the efficiency of genome editing.^[4,5,7,11]

Encapsulation, e.g., within a lipid nanoparticle or a polymer particle, could overcome these problems.^[4,5,7,11]

Serum Protein Interactions

Upon systemic injection, delivery nanoparticles immediately encounter circulating plasma. These interactions may result in the creation of a protein corona, which alters the physical/chemical properties of the nanoparticles, their biodistribution, cell uptake pathways, and clearance routes. The content of the protein corona thus influences the probability that a nanoparticle is eliminated by the immune system or successfully targeted, and needs to be controlled to achieve effective delivery of CRISPR/Cas9.^[4,5,7,11]

Blood-Tissue and Extracellular Matrix Barriers

When in circulation, CRISPR delivery vehicles must overcome the obstacle of breaching the vasculature and penetrating the extracellular matrix barrier to reach target cells. Large macromolecule-delivering systems can face difficulty passing between the circulation and the tissue space due to the junctions at the endothelium lining some tissues. In addition, a tightly knit extracellular matrix may hinder the distribution of nanoparticles in the interstitial fluid, reducing their penetration depth into the tissue and therefore hindering effective treatment of solid tumors and other tissues.^[4,5,7,11]

Cellular uptake

Cellular uptake is a crucial step for the success of CRISPR/Cas9 processes, as the therapeutic payload must cross the cellular membrane to function. The relatively

large size, negative charge, and hydrophilic properties of plasmid DNA, mRNA and Cas9/gRNA ribonucleoproteins (RNPs) make passage across the lipid bilayer unlikely. Consequently, entry into cells occurs via endocytosis or other methods of membrane transduction. Low cellular entry reduces the number of CRISPR/Cas9 factors within cells and therefore reduces genome-editing efficacy.^[4,5,7,11]

Endocytosis-Mediated Uptake

The plasma membrane is a major challenge to overcome for the CRISPR/Cas9 components. Naked DNA and RNA are negatively charged molecules that interact poorly with the hydrophobic lipid core of the cell membrane. At the same time, the Cas9-gRNA RNP complex is too large to penetrate the cell membrane on its own. For this reason, most delivery systems utilize positively charged or ionizable lipids, polymers, peptides, or other molecules that help introduce these complexes into the cell.^[4,5,7,11]

Endosomal Entrapment

Although cellular uptake can be efficient, a significant amount of the delivered CRISPR cargo can end up enclosed within endosomes. Because endosomes undergo an acidic environment in which their contents are often digested by enzymes, it is crucial that cells internalize the CRISPR cargo in a manner that allows its release from endosomes. Otherwise, the CRISPR complex will be trapped in endosomes and delivered to lysosomes, rendering genome editing impossible.^[4,5,7,11]

Endosomal confinement is the primary reason for reduced efficacy of CRISPR/Cas9 therapeutics delivered via lipid nanoparticles, polymeric nanoparticles, liposomes and other nanodelivery systems that rely on endocytosis for cell internalization. Most nanoparticles are internalized through endocytosis but must then be trafficked intracellularly to deliver the molecular payload to the correct site in the genome and edit the DNA. If nanoparticles fail to escape endosomes promptly, they are rapidly targeted to lysosomes and degraded, leading to greatly diminished genome editing efficiency.^[4,5,7,11]

Formation and Maturation of Endosomes

Upon reaching and entering a cell, nanodevices are engulfed by the cell, forming early endosomes. As the pH within these early endosomes decreases, they develop into late endosomes. Late endosomes can also be found fused with lysosomes, through which DNA, RNA and proteins can be degraded by various enzymes. Only a minimal fraction of RNA/Cas9 can hence reach the cytoplasm to carry out its function.^[4,5,7,11]

Degradation of CRISPR Cargo

The pathway of lysosomal and endosomal degradation is a major obstacle in CRISPR/Cas9-based therapies. mRNA and guide RNA can be degraded by nucleases, and Cas9 can be degraded by proteases. Plasmid DNA may also be broken down before it can reach target sites

in the nuclei of recipient cells. The result is lower intracellular concentrations of active CRISPR reagents and thus lower gene editing efficiency.^[4,5,7,11]

Endosomal Escape Mechanisms

Delivery systems can be engineered to promote endosomal escape by a variety of mechanisms. For instance, pH-responsive polymers or lipids can be used that undergo a conformational change or charge neutralization in the acidic endosomal environment, thereby disrupting the endosomal membrane. Another strategy is to use ionizable lipids that become protonated at acidic pH and interact with the anionic endosomal membrane, also causing its destabilization.^[4,5,7,11]

Membrane-disrupting peptides, fusogenic lipids, polymers, and osmotic pressure have also been explored as means of promoting endosomal escape by increasing membrane permeability or promoting fusion of endosomes with other membranes. These approaches allow CRISPR complexes to reach the cytoplasm.^[4,5,7,11]

Balance Between Escape and Toxicity

Although increasing the level of endosomal escape could potentially enhance CRISPR delivery, too great an extent of membrane disruption can be detrimental to cells, leading to cytotoxicity. Moreover, highly cationic or membrane-disruptive moieties can engage in undesired interactions with cellular membranes and proteins. Thus, a successful delivery system should maximize endosomal escape while minimizing membrane disruption and, consequently, the elicitation of an undesired inflammatory response.^[4,5,7,11]

Intracellular Trafficking

Intracellular movement of delivered molecules can be one of the most limiting stages of CRISPR/Cas9 genome editing. Although endocytosis of CRISPR/Cas9 complexes with appropriate delivery vehicles has been extensively explored, the journey of the delivered molecule through intracellular pathways remains essential for the success of genome editing and has, as yet, proven challenging. There are multiple steps after delivery for efficient intracellular transport of CRISPR/Cas9 agents, influenced by factors such as the properties of the delivery vehicle and cargo, the interaction of these elements with intracellular factors, and the cell type in which the delivered complex will act. The CRISPR/Cas9 therapeutic cargo must undertake complex trips through different cellular organelles following its transport into the cell upon endocytosis. All delivered CRISPR/Cas9 complexes end up inside cellular organelles called endosomes and, with time, reach late endosomes and finally lysosomes, where most of them are destroyed. The CRISPR/Cas9 cargo must escape this endosomal trafficking to reach its target – a stage known as endosomal escape – and once it is out of the endosome and into the cytoplasm, it must continue to travel until it finally reaches the nucleus. A successful delivery of CRISPR/Cas9 therefore involves an efficient

transition across endosomal barriers, through cytoskeletal transport, to nuclear entry.^[4,5,7,11]

The cytoskeletal framework acts as a road network for organelles and larger molecular assemblies to reach their target site within the cell. The cytoskeleton (including microtubules, intermediate filaments and actin filaments, along with associated motor proteins) provides dynamic roadways that assist effective intracellular delivery of a vast majority of delivered molecular components. CRISPR/Cas9 delivered within some vehicles interacts with different parts of the cytoskeletal structure, often by connecting to motor proteins such as kinesin and dynein along the microtubules, and myosin acting in conjunction with actin filaments. Actin, for example, has been shown to play a crucial role in intracellular movement of endosomes harbouring the delivered CRISPR/Cas9 complex toward the nuclear destination; likewise, dyneins act along the microtubules towards the negative ends, moving towards the centre and the source of the original movement.^[4,5,7,11]

While interactions with the cytoskeletal framework help speed up delivery, in certain situations they can also inhibit efficient transport of CRISPR/Cas9 cargo. The precise shape, size, overall charge, hydrophobicity and other physical and chemical properties of the delivered package influence delivery and internal movement of the cargo. For example, larger molecules find it more difficult to travel efficiently through the cellular structure due to increased steric limitations in a crowded interior. Charge is another factor to be considered, since delivery vehicles with positive surface charge facilitate entry through membranes and encourage interaction with cellular elements and molecules inside the cell, supporting fast, non-specific endocytosis and increasing the likelihood of cell entry via interaction with cell surface membranes that carry a net negative charge. However, since many intracellular components also carry a net negative charge, a positive surface charge also tends to encourage unwanted sequestration of the delivery vehicle by internal cellular protein factors. The properties of the cargo itself are also crucial, particularly when the cargo is in a non-functional and non-directly translated form. Thus, delivering a DNA plasmid requires many more steps – endosomal escape, unpacking in the cytoplasm, transport into the nucleus, and finally transcription into mRNA, which is in turn translated into Cas9 protein together with the guide RNA. mRNA only needs to escape the endosome to achieve translation into Cas9 protein, although there is potential for lower delivery efficiency since mRNA is less stable than DNA.^[4,5,7,11]

Delivery of RNPs means all components are ready to work; they need only to escape the endosome and begin mediating genomic modification. The nature of the CRISPR/Cas9 component therefore determines the most effective mechanism of intracellular delivery.^[4,5,7,11]

There are a number of ways of escaping the endosome into the cytoplasm, from where the delivered molecules should ideally be targeted towards the nucleus, where the target DNA is located. A broad range of techniques, taking advantage of membrane destabilization induced by delivery agents, acidification-driven changes in endosomal membrane conformation, swelling of endosomes followed by physical disruption of the endosomal membrane (osmotic lysis), and vesicle rupture followed by fusion, are used to make delivery effective. Effective endosomal escape strategies for delivering CRISPR/Cas9 molecules facilitate transport of the cargo, making this the most critical step required before efficient internal transport of the complex and its entry into the nucleus, where it exerts its effect, can be achieved.^[4,5,7,11]

Protein interactions with delivery vehicles can influence intracellular delivery by forming a protein corona around the vehicle that alters its trajectory. Some protein interactions assist intracellular transport of delivery vehicles, while others hinder it. Ultimately, understanding how a delivered molecule interacts with internal proteins within the cellular milieu provides insight for design strategies that deliver complexes to target organelles with specificity.^[4,5,7,11]

Stabilization of the delivered CRISPR/Cas9 complex within the delivery vehicle is of prime concern. The delivery vehicle needs to protect the cargo from degradation in hostile intracellular environments, while also being able to break up and release the cargo when and where required for maximum efficiency of the desired genomic modification. Intracellular trafficking of delivery vehicles therefore needs to be well timed to maintain the stability of the delivered material before and after release. Different tissues, cell lines and even individuals vary in their internal cellular infrastructure for taking up delivered agents. Thus, not only delivery to specific target cells, but also the delivery dynamics for varying cell types, must be considered.^[4,5,7,11]

Likewise, cellular factors controlling trafficking can differ as cells differentiate; for example, delivery into a stem cell will necessarily differ from delivery into a differentiated neuron. Overall, cell heterogeneity is an important consideration affecting the efficiency of intracellular delivery of agents.^[4,5,7,11]

Nuclear delivery

Nuclear delivery is a necessary step for effective intracellular targeting and processing of CRISPR/Cas9 therapeutics. Once CRISPR/Cas9 complexes have been successfully delivered into cells without undergoing degradation during endocytosis, they must further be transported into the nucleus to perform their function of targeting and editing the desired gene, since all genomic DNA resides in the nucleus. The outcome of genomic editing relies heavily on the nuclear delivery process, which is especially important when the constructs used

are plasmid DNA or Cas9–guide RNA, as both require nuclear localization for their function to be fulfilled.

As the type of CRISPR/Cas9 system varies, so do the pathways for its nuclear delivery. Plasmid DNA, being a high-molecular-weight material, is usually transferred into the nucleus through the nuclear pore complex. Cell division appears to enhance migration into the nucleus due to transient breakdown of the nuclear envelope, which may also explain why dividing cells are usually more sensitive to CRISPR/Cas9 delivered via plasmids. Meanwhile, utilizing RNPs or mRNAs reduces Cas9 persistence within cells and its interaction with the genome, enabling highly specific targeting, though efficient translocation into the nucleus can still be a constraint.^[4,5,7,11]

The nuclear pore complex (NPC) serves as the key channel for active or passive transport of macromolecules between the cytoplasm and the nucleus. Molecules up to about 40 kDa can diffuse passively through the NPC without requiring energy; molecules of higher molecular weight require active assistance to be translocated into the nucleus. In this case, adding nuclear localization signals (NLSs) to Cas9 assists its active translocation through the nuclear pore complex via an energy-dependent mechanism, whereby the NLS of the transported molecule is specifically recognized by nuclear import receptor proteins such as importin- α/β . Engineering of NLSs into the Cas9 protein can therefore accelerate its delivery into the nucleus, promoting editing efficiency through greater nuclear accumulation. It has further been reported that the number and position of NLSs affect their affinity for importin- α/β and hence mediate transport of the protein into the nucleus, followed by its association with guide RNA and target DNA.^[4,5,7,11]

Cargo degradation

Cargo can be degraded by various intracellular processes, decreasing the efficacy of delivery. After endocytosis, therapeutic cargo – including plasmid DNA, Cas9 mRNA, gRNA, and Cas9 RNP – can be subjected to nuclease activity, proteolysis, low pH environments, and other degradative processes that lower its bioavailability. Hence, only a small amount of delivered CRISPR/Cas9 cargo is estimated to reach its intracellular target site. Nucleic-acid-based CRISPR cargos are very fragile: plasmid DNA can be cut by cellular nucleases, while mRNA and gRNA are vulnerable to ubiquitous enzymes. Degradation of RNA molecules can lead to diminished accumulation of Cas9 protein and guide RNA necessary for performing genome editing, lessening the quantity of Cas9–gRNA complexes formed to carry out targeted edits. A further factor in the use of RNP is the potential for Cas9 protein degradation; protein-degrading enzymes can break down the Cas9 protein, meaning fewer Cas9–guide RNA–target RNA complexes can form, resulting in lower editing functionality and efficiency. This can also be an advantage, since a short

half-life allows removal of functional protein, which inhibits off-target effects of Cas9. Therefore, care must be taken to have enough Cas9 present for editing to occur, but not so much that it remains in the cell after the edit has taken place.^[4,5,7,11]

5. NON-VIRAL DELIVERY SYSTEMS FOR CRISPR/CAS9

The main challenge in engineering non-viral CRISPR/Cas9 delivery vehicles concerns the need to balance multiple functions: shielding the therapeutic cargo against degradation while promoting efficient cellular uptake, endosomal escape, intracellular trafficking and nuclear delivery. An optimal non-viral system should therefore shield the CRISPR/Cas9 cargo, enhance its cellular internalization, promote its escape from endosomes, and deliver it to the desired intracellular target with minimal toxicity.^[4,5,7,11]

Overview of non-viral delivery approaches

Non-viral methods offer an excellent avenue for the delivery of CRISPR/Cas9 components. These comprise native, synthetic or semi-synthetic polymers with the capacity to hold plasmid DNA, mRNA, gRNAs, and Cas9 RNPs. Depending on the type of CRISPR component used, the target cell, the route of administration, how long Cas9 should remain active, and the overall purpose of using CRISPR/Cas9, the appropriate delivery system is selected.^[4,5,7,11]

Advantages over viral vectors

The major advantages of non-viral delivery systems are reduced risk of genomic integration, lower vector-associated immunogenicity, flexible cargo choice, transient expression of CRISPR/Cas9 components, tunable physicochemical properties, opportunity for targeted delivery, and ease of manufacturing. These advantages make non-viral delivery systems appealing for developing safer and more versatile RNAi- or CRISPR/Cas9-based therapeutics.^[4,5,7,11]

Limitations of conventional non-viral systems

The major limitations of conventional non-viral systems include low efficiency of delivery to target cells or tissues, low cellular uptake, endosomal entrapment, degradation of delivered molecules within the cell, insufficient tissue specificity, inefficient intracellular trafficking and nuclear delivery, potential cytotoxicity, inflammatory response, formulation instability, and poor extrapolation from in vitro to in vivo studies. Overcoming these barriers requires developing multifunctional and rationally designed delivery systems that can surmount several of these challenges simultaneously.^[4,5,7,11]

Role of nanotechnology in overcoming delivery barriers

Nanotechnology offers multifunctional approaches for overcoming the key challenges in CRISPR/Cas9 delivery. By offering simultaneous solutions to related problems such as cargo protection, targeted delivery,

endosomal escape, regulated release, intracellular trafficking, and nuclear targeting, nanocarriers can significantly enhance the efficiency and therapeutic safety of genome-editing treatments. Further developments in nanotechnology, which provide opportunities for engineering nanomaterials with improved design features for targeted delivery of genome-editing machinery, will be critical for the clinical translation of CRISPR/Cas9 technology.^[4,5,7,11]

6. NANOTECHNOLOGY-BASED DELIVERY PLATFORMS

Novel approaches to drug delivery, including for CRISPR/Cas9, increasingly revolve around nanotechnology-based delivery systems. These nanoparticles can protect the genes or therapeutics used for gene editing, allowing them to enter cells. The physical and chemical characteristics of the nanoparticle can be tuned to control where and how it enters the cell, the method by which therapeutic contents escape from endosomes, and the transport of therapies to target tissues. The physical and chemical properties that can be manipulated include size, surface charge, composition, shape and surface chemistry.^[5,6,8,11,12]

Lipid nanoparticles

Lipid nanoparticles (LNPs) are the most explored nanotechnology-based systems for the delivery of CRISPR/Cas9. LNPs can effectively deliver Cas9 mRNA and gRNA by encapsulating the genetic material inside the nanoparticle. Ionizable lipids show great potential for promoting packaging of the RNA cargo and endosomal escape through disruption of the endosomal membrane. LNPs have proven promising for the temporary delivery of Cas9 mRNA and gRNA, while tissue-specific targeting and the ability to deliver outside the liver remain unaddressed challenges.^[5,6,8,11,12]

Polymeric nanoparticles

Polymeric nanoparticles offer an additional versatile platform for CRISPR/Cas9 delivery. Synthetic polymers such as polyethyleneimine, PLGA, and chitosan can be used for binding or encapsulating nucleic acids or proteins. The properties of these polymers can be tuned by modifying their chemical structure to enhance features such as cargo protection, cellular uptake, endosomal escape, and triggered release of the payload. Nevertheless, the application of cationic polymers is limited due to their high toxicity, which must be minimized by tuning their physicochemical properties.^[5,6,8,11,12]

Lipid-polymer hybrid nanoparticles

Lipid-polymer hybrid nanoparticles have been designed to combine characteristics of both lipid- and polymer-based systems. These carriers combine the cargo-loading capacity and structural integrity of polymers with the cell-penetrating and endosome-escaping properties of lipids, enabling the design of bifunctional systems for

targeted delivery and controlled release of various CRISPR/Cas9 cargoes.^[5,6,8,11,12]

Dendrimers

Dendrimers are generally the most highly branched nanostructures, possessing numerous surface groups. Their surface can be modified to enable complexation with nucleic acids, forming nanostructure complexes. They can be tailored for increased biocompatibility, cell uptake and intracellular delivery. Nonetheless, excess positive or negative surface charge can lead to cell death.^[5,6,8,11,12]

Inorganic nanoparticles

Inorganic nanoparticles, including gold and silica nanoparticles, have also been explored for CRISPR/Cas9 delivery. These materials can be easily surface-modified with nucleic acids, proteins, or peptides to create complexes with good stability and multifunctionality, but their biodegradability, bioaccumulation, and potential toxicity should be carefully considered.^[5,6,8,11,12]

7. NANOCARRIER DESIGN STRATEGIES FOR EFFICIENT CRISPR/CAS9 DELIVERY

The choice of cargo and its loading method is a key factor to consider when designing nanocarriers, since the carrier will be loading and delivering CRISPR/Cas9 in the form of plasmid DNA, mRNA, guide RNA, or formed ribonucleoproteins. The nanocarrier should be capable of carrying the therapeutically required amount of cargo while maintaining its biological integrity. Encapsulation and loading of CRISPR/Cas9 components can be achieved using traditional procedures such as precipitation, electrostatic complexation and surface conjugation, depending on the nature of the cargo.

Size and morphology of the nanocarrier are also important factors affecting the biodistribution of the system, and are key parameters in determining the efficiency of cellular entry, tissue distribution and intracellular trafficking. When designing the nanocarrier, an appropriate size needs to be chosen based on the intended route of administration, and carrier morphology, which further affects interaction with cellular membranes and in vivo biodistribution, must be optimized alongside size. Cellular uptake and tissue penetration tend to decrease with increasing particle size above 200 nm, while particle sizes below about 20 nm can be rapidly cleared from the body.^[9,11,12,13,14]

The charge on the nanocarrier is also a vital parameter influencing the biodistribution of the delivery system. For nucleic acid (including dsDNA and mRNA) encapsulation, positively charged carriers show greater affinity for these negatively charged biomolecules, effectively encapsulating and carrying them while maintaining integrity, and promoting release inside the cell due to the higher affinity between negatively charged cellular membranes and positively charged carrier surfaces. However, negatively charged carriers

cause less toxicity and less nonspecific binding with cells than positively charged nanocarriers, and tend to remain longer in circulation.^[9,11,12,13,14]

The toxicity and rapid blood clearance associated with positively charged carriers can be mitigated by incorporating pH-sensitive ionizable groups, which become positively charged under the acidic pH of the cell/endosome. Similarly, negatively charged systems tend to avoid nonspecific interactions and toxicity while delivering DNA/RNA into cells.^[9,11,12,13,14]

Endosomal escape is an important function to consider when designing a system to deliver CRISPR/Cas9 ribonucleoproteins, since cargo delivered into the cell after endocytosis via passive pathways needs to be released from endosomes into the cytoplasm/nucleus for activity and proper function. Endosomal escape should be facilitated by introducing membrane-active elements, such as polymers, peptides and lipids, into the nanocarrier design. These rupture or destabilize the endosomal membrane, allowing liberation of contents directly into the cell and avoiding the degradative environment, so that the cargo's effects can be exerted on target cells and tissues.^[9,11,12,13,14]

Targeting ligands presented on the nanocarrier surface can enhance cellular/tissue targeting and the efficiency of cargo delivery. Various targeting moieties, including antibodies, peptides, aptamers and carbohydrates, can be conjugated onto nanocarriers to specifically recognise target cell receptors, thereby increasing intracellular delivery efficiency and minimizing toxicity from off-target actions; however, care is needed since receptor expression varies greatly from tissue to tissue, so the choice of ligand should be tissue-specific.^[9,11,12,13,14]

Stimuli-responsive nanocarrier systems for CRISPR/Cas9 generally remain intact, responding to triggers based on intracellular pH change and redox potential, until they reach and enter the target cell. Once inside, environmental changes trigger collapse or degradation of the nanocarrier, leading to release of the payload. This avoids premature degradation and increases delivery efficiency into target tissue cells. Finally, materials used to create nanocarriers should fulfil the criteria of biocompatibility and biodegradability to allow processing of by-products from the body after their functional outcome. The rate of biodegradation is a critical consideration depending on the route of administration, and biocompatibility is particularly important for systemic targeting applications, while biodegradability is particularly important for local targeted applications.

Designing an efficient CRISPR/Cas9 nanocarrier is therefore a sophisticated, multi-phase, multidisciplinary process that is application-specific, requiring optimization of cargo loading, particle size, surface charge, targeting, endosomal escape, endocytosis,

intracellular release, biodegradability, controllability and manufacturability. Future studies on CRISPR/Cas9 nanocarriers will also need to focus on targeting strategies and stimuli-responsive design to ensure that gene-editing machinery specifically and efficiently targets the intended cells or tissue and releases its payload based on the appropriate stimuli, with minimal potential for toxicity and off-target editing.^[9,11,12,13,14]

8. CURRENT PROGRESS AND RECENT ADVANCES

Recent advances suggest a shift from simple CRISPR/Cas9 carrier systems to precision-designed, targeted, multifunctional, and stimuli-responsive nanocarriers. The future of the field will be shaped by strategies to achieve efficient extrahepatic delivery and tissue-specific targeting, as well as reduction of toxicity and immunogenicity and standardization of manufacturing and safety assessment. These advances will determine the clinical viability of non-viral CRISPR/Cas9 delivery and therapies.^[9,11,13,14,15]

Delivery of Cas9 plasmid DNA

While delivering plasmid DNA encoding the CRISPR/Cas9 system offers a relatively simple, flexible, and inexpensive method for genome editing, its applications are limited by substantial cargo size restrictions, inefficient delivery into cells and the nucleus, endosomal sequestration, delayed induction of edits, prolonged Cas9 persistence, and potential insertional mutagenesis. Consequently, significant efforts are being made to develop targeted lipid-, polymeric-, hybrid- and other nanoparticle-based systems that enhance cargo stability, promote endosomal escape and intracellular trafficking, and reduce toxicity and off-target genome editing effects.^[4,7,8,11]

Delivery of Cas9 mRNA

Cas9 mRNA delivery represents a transient, efficient, and versatile alternative to plasmid DNA delivery. Its main advantage is that it allows cytoplasmic translation, bypassing the need for transcription of the Cas9 gene in the nucleus. Moreover, compared to DNA, Cas9 mRNA typically results in faster production of the Cas protein and a shorter persistence of nuclease activity. However, mRNA instability, inefficient cellular delivery, endosomal sequestration, tissue-specific barriers to delivery, and the risk of an undesired immune response constitute major challenges for its clinical application. The development of safer and more efficient delivery systems based on targeted and biodegradable nanoparticles, including LNPs, will be critical for the successful clinical translation of Cas9 mRNA-based genome editing.^[9,11,12,13,14]

Delivery of Cas9 RNP

In comparison to plasmid-based systems, delivery of RNP is rapid in target cells and is associated with increased genome editing efficiency, transient expression of Cas9 nuclease, lower overall Cas9 exposure, and no

requirement for transcription of plasmid DNA, avoiding its potential genotoxic consequences. However, delivery of RNP into target cells across several barriers remains a significant challenge. The future of RNP-based CRISPR/Cas9 genome editing lies in the development of biodegradable, targeted nanocarriers that can optimize RNP function by facilitating stability in circulation, cell entry, endosomal escape, tissue targeting, and intracellular release while maintaining Cas9 activity. This will support the transition from preclinical and ex vivo applications of CRISPR/Cas9 towards clinical settings.^[9,11,12,13,14]

Current clinical and translational progress

Clinical and translational progress indicates a gradual transition from conventional viral and ex vivo approaches toward transient, targeted, and increasingly non-viral in vivo delivery. Among non-viral approaches, lipid nanoparticles (LNPs) appear to be the most clinically advanced and are therefore likely to play a major role in liver-directed therapeutics. Nevertheless, achieving safe and effective extrahepatic targeting, improving targeting specificity, fine-tuning immune activation, and establishing manufacturing consistency represent key areas for future research and development. Similarly, further optimization of different nanocarriers will be critical for maximizing the therapeutic potential of CRISPR/Cas9 and the novel genome editors of the future.^[11,13,16,17]

9. SAFETY AND TOXICOLOGICAL CONSIDERATIONS

The safety of CRISPR/Cas9 therapeutics can be achieved through balancing several factors, including delivery efficiency, precision of editing, transient expression, tissue targeting, and biocompatibility. The use of biodegradable nanocarriers, rational design strategies, optimization of Cas9 variants, and the use of transient RNP or mRNA may reduce toxicity and unwanted genome editing. Long-term safety studies and standardized toxicological assessments are critical to ensure the successful clinical implementation of CRISPR/Cas9 delivery systems.^[7,11,12,15,17]

10. TRANSLATIONAL CHALLENGES

Successful clinical translation of CRISPR/Cas9 technology requires more than high editing efficiency in cell culture. Future therapies will need to demonstrate tissue-specific targeting, efficient delivery, transient expression of Cas9, low toxicity, absence of off-target effects, a consistent manufacturing process, and long-term safety. The research field is moving towards multifunctional non-viral systems that can provide multiple layers of control over the therapeutic genome editing process, addressing the combinatorial interventions required to effectively treat many diseases in the clinic.^[11,13,14,15,17]

11. FUTURE PERSPECTIVES

The future of CRISPR/Cas9 delivery systems depends on the creation of highly sophisticated, targeted, biodegradable, multifunctional non-viral systems, in which therapeutic efficiency is increased through the use of smart nanomaterials, transient RNA or DNA delivery, tissue-specific targeting, multifunctional design, and improved manufacturing. These research efforts are expected to make an essential contribution to the continued development of CRISPR/Cas9 into a viable therapeutic modality.^[11,13,14,15,17]

12. UPDATED CLINICAL AND TRANSLATIONAL PERSPECTIVE

Clinical translation of CRISPR/Cas9 delivery should distinguish between ex vivo genome-editing therapies and direct in vivo administration. The regulatory success of CRISPR-based medicine is important evidence that genome editing can reach patients, but the first approved CRISPR therapy, exagamglogene autotemcel (Casgevy), is an ex vivo cell-based treatment for sickle cell disease and transfusion-dependent β -thalassemia rather than a systemic nanocarrier administered directly to the patient. Consequently, the central translational question for nanotechnology-enabled non-viral delivery remains how to achieve reproducible, tissue-selective in vivo editing while limiting systemic exposure and unintended genomic modification.^[11,12,13]

A landmark example of in vivo non-viral editing is NTLA-2001, an investigational lipid-nanoparticle formulation carrying Cas9 mRNA and a guide RNA targeting the transthyretin gene. In an early phase 1 study, intravenous administration produced dose-dependent reductions in serum transthyretin, providing clinical proof of concept for systemic LNP-mediated CRISPR/Cas9 editing in humans. Longer-term phase 1 data reported in 2025 showed that a single administration

of nexiguran ziclumeran (nex-z; formerly NTLA-2001) produced deep and durable reductions in serum transthyretin in patients with hereditary transthyretin amyloidosis with polyneuropathy. These results are encouraging, but they also illustrate the importance of careful patient selection, organ tropism, immune monitoring and long-term surveillance.^[16,17]

Current delivery research is therefore moving beyond simple encapsulation toward rational control of particle composition, size, surface chemistry, ionization behaviour, protein-corona interactions, cellular uptake and intracellular release. Non-liver delivery is particularly important because many LNP formulations preferentially accumulate in the liver after systemic administration. Recent studies have explored selective-organ-targeting concepts, ligand-mediated targeting, lipid-library screening, biodegradable materials and alternative carriers such as polymers, extracellular vesicles and engineered virus-like particles. These approaches may broaden the range of tissues that can be reached, but each introduces additional formulation, pharmacokinetic, immunological and manufacturing variables.^[13,14,15]

A translationally useful nanocarrier should therefore be evaluated as an integrated drug-delivery system rather than only by its editing percentage in a cell culture model. Important quality attributes include particle size and polydispersity, encapsulation or complexation efficiency, cargo integrity, surface composition, colloidal stability, release behaviour, sterility, endotoxin burden, potency, tissue distribution and batch-to-batch reproducibility. For genome editing, these pharmaceutical attributes must be linked to biological endpoints such as on-target editing, off-target editing, editor persistence, inflammatory responses and organ toxicity.^[5,8,11,13]

Table 1: Comparative Features of CRISPR/Cas9 Therapeutic Cargo Formats.

Cargo	Major advantages	Major limitations	Typical delivery requirement	Therapeutic implication
Plasmid DNA	Stable and scalable; can encode Cas9, guide RNA and donor template	Large size; nuclear entry required; prolonged expression; innate immune concerns	Polymeric, lipid or hybrid carriers; efficient nuclear access	Useful when sustained expression or multi-component delivery is required
Cas9 mRNA + sgRNA	Rapid cytoplasmic translation; transient editor exposure; no DNA expression cassette	RNA instability; endosomal escape and tissue targeting remain challenging	Lipid nanoparticles and other RNA-compatible nanocarriers	Attractive for transient in vivo editing and liver-directed applications
Cas9-sgRNA RNP	Immediate activity; short intracellular persistence; avoids transcription/translation	Protein/RNA complex is bulky and difficult to transport across membranes	Lipid/polymer systems, electroporation, peptides, vesicles or VLPs	Well suited to controlled, transient editing, particularly ex vivo and emerging in vivo applications

Table 2: Comparison of Major Nanotechnology-Enabled Non-Viral CRISPR/Cas9 Delivery Platforms.

Platform	Cargo compatibility	Key strengths	Key limitations	Translational considerations
Lipid nanoparticles	mRNA, sgRNA, RNP; some DNA formats	High RNA-loading efficiency; established manufacturing experience; tunable ionizable lipids	Liver bias for many formulations; innate immune activation; endosomal escape inefficiency	Most clinically advanced non-viral platform; strong potential for organ-selective engineering
Polymeric nanoparticles	DNA, mRNA, sgRNA, RNP	Chemical versatility; controlled degradation; tunable charge and functionality	Potential cytotoxicity, heterogeneous formulations and variable in vivo performance	Biodegradability and reproducible scale-up are critical
Lipid–polymer hybrids	DNA, RNA and protein cargos	Combines structural stability with lipid-mediated uptake and release	More complex composition and manufacturing	Useful when single-material systems cannot balance protection and release
Dendrimers	Nucleic acids and proteins	Highly branched structure; dense functional surface; precise modification	Cationic toxicity and accumulation may limit use	Requires careful control of surface charge and biodegradation
Inorganic nanoparticles	DNA, RNA, proteins and RNPs	High surface functionalization capacity; imaging/theranostic possibilities	Potential persistence, bioaccumulation and material-specific toxicity	Strong preclinical potential but additional safety and clearance data are needed
Extracellular vesicles / VLPs	Protein, RNA and RNP cargos	Biological interfaces and potential for efficient protein delivery	Loading, characterization, scalability and product consistency	Promising emerging approaches requiring robust manufacturing standards

13. KEY GAPS THAT MUST BE ADDRESSED BEFORE BROAD CLINICAL ADOPTION

Tissue and cell specificity: Systemic nanoparticles must reach the correct tissue and, ideally, the correct cell population. Liver tropism is useful for hepatic targets but is not sufficient for diseases of the central nervous system, skeletal muscle, lung, immune system or solid tumours.^[11,12,14,15,17]

Editor exposure and off-target activity: Transient mRNA or RNP delivery can shorten the period of nuclease exposure, but transient delivery does not eliminate off-target risk. Guide-RNA design, high-fidelity Cas variants and genome-wide safety assessment remain essential.^[11,12,14,15,17]

Endosomal escape: A large fraction of internalized nanocarriers can remain trapped in endosomal compartments. Increasing escape without damaging the plasma or endosomal membranes is a central formulation challenge.^[11,12,14,15,17]

Manufacturing and quality control: Clinical products require reproducible composition, potency, sterility, stability and scalable manufacturing. Multicomponent

nanoparticles may create additional critical quality attributes and analytical burdens.^[11,12,14,15,17]

Long-term safety: Genome editing can create durable genomic changes; therefore, safety assessment must extend beyond short-term toxicity to include on-target structural variation, unintended edits, immune responses and long-term clinical follow-up.^[11,12,14,15,17]

14. CONCLUSION

Nanotechnology-enabled non-viral delivery has become a major enabling technology for the therapeutic development of CRISPR/Cas9 genome editing. The choice among plasmid DNA, mRNA and RNP cargoes must be aligned with the required duration of editor activity, target tissue, route of administration and desired editing outcome. Lipid nanoparticles currently provide the clearest clinical precedent for systemic in vivo delivery, whereas polymeric, hybrid, inorganic, vesicular and virus-like systems provide complementary opportunities for cargo protection, targeting and controlled release. Despite rapid progress, efficient extrahepatic targeting, endosomal escape, tissue specificity, off-target control, immunogenicity, scalable manufacturing and long-term safety remain significant barriers. Future progress will depend on integrating

pharmaceutical formulation science with genome-editor engineering, quantitative biodistribution studies, predictive safety assessment and clinically relevant potency assays. Well-characterized, biodegradable and multifunctional nanocarriers that deliver transient editing machinery to precisely selected cells may ultimately provide a practical route toward safer and more effective CRISPR/Cas9 therapeutics.^[11,12,14,15,17]

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ETHICAL STATEMENT

This article is a literature review and does not involve new experiments involving human participants or animals.^[4,7,8,11]

DECLARATIONS

Conflict of Interest: All six authors confirm that they have no conflict of interest or competing interest related to this manuscript. The conflict-of-interest declaration has been reviewed and verified by all six authors.

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REFERENCES

- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, 2012; 337(6096): 816–821. doi:10.1126/science.1225829.
- Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science*, 2013; 339(6121): 819–823. doi:10.1126/science.1231143.
- Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. *Science*, 2014; 346(6213): 1258096. doi:10.1126/science.1258096.
- Li L, Hu S, Chen X. Non-viral delivery systems for CRISPR/Cas9-based genome editing: challenges and opportunities. *Biomaterials*, 2018; 171: 207–218. doi:10.1016/j.biomaterials.2018.04.031.
- Givens BE, Naguib YW, Geary SM, Devor EJ, Salem AK. Nanoparticle-based delivery of CRISPR/Cas9 genome-editing therapeutics. *AAPS J.*, 2018; 20(6): 108. doi:10.1208/s12248-018-0267-9.
- Gong Y, Tian S, Xuan Y, Zhang S. Lipid and polymer mediated CRISPR/Cas9 gene editing. *J Mater Chem B.*, 2020; 8(20): 4369–4386. doi:10.1039/D0TB00207K.
- Behr M, Zhou J, Xu B, Zhang H. In vivo delivery of CRISPR-Cas9 therapeutics: progress and challenges. *Acta Pharm Sin B.*, 2021; 11(8): 2150–2171. doi:10.1016/j.apsb.2021.05.020.
- Ashok B, Peppas NA, Wechsler ME. Lipid- and polymer-based nanoparticle systems for the delivery of CRISPR/Cas9. *J Drug Deliv Sci Technol*, 2021; 65: 102728. doi:10.1016/j.jddst.2021.102728.
- 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(5): 1500–1519. doi:10.1007/s13346-023-01320-z.
- Du Y, Liu Y, Hu J, Peng X, Liu Z. CRISPR/Cas9 systems: delivery technologies and biomedical applications. *Asian J Pharm Sci.*, 2023; 18(6): 100854. doi:10.1016/j.ajps.2023.100854.
- Madigan V, Zhang F, Dahlman JE. Drug delivery systems for CRISPR-based genome editors. *Nat Rev Drug Discov*, 2023; 22(11): 875–894. doi:10.1038/s41573-023-00762-x.
- Kim M, Hwang Y, Lim S, Jang H-K, Kim H-O. Advances in nanoparticles as non-viral vectors for efficient delivery of CRISPR/Cas9. *Pharmaceutics*, 2024; 16(9): 1197. doi:10.3390/pharmaceutics16091197.
- Masarwy R, Stotsky-Oterin L, Elisha A, Hazan-Halevy I, Peer D. Delivery of nucleic acid based genome editing platforms via lipid nanoparticles: clinical applications. *Adv Drug Deliv Rev.*, 2024; 211: 115359. doi:10.1016/j.addr.2024.115359.
- Wu F, Li N, Xiao Y, et al. Lipid nanoparticles for delivery of CRISPR gene editing components. *Small Methods*, 2026; 10(2): 2401632. doi:10.1002/smt.202401632.
- Yang J, Chen Y, Chen X, Ping Y. Non-viral delivery of genome-editing tools for treatment of genetic disorders. *Acta Pharm Sin B.*, 2026; 16(5): 2903–2928. doi:10.1016/j.apsb.2026.02.014.
- Gillmore JD, Gane E, Täubel J, et al. CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. *N Engl J Med.*, 2021; 385(6): 493–502. doi:10.1056/NEJMoa2107454.
- Gillmore JD, Gane E, Täubel J, et al. Nexiguran ziclumeran gene editing in hereditary ATTR with polyneuropathy. *N Engl J Med*, 2025; 393(14): 1375–1386. doi:10.1056/NEJMoa2510209.