International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

ADVANCES IN CRISPR-CAS9 DELIVERY: A COMPARATIVE REVIEW OF PHYSICAL AND NANOPARTICLE-BASED METHODS

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ABSTRACT:

The CRISPR-Cas9 system has transformed genome editing because of its ease of use, accuracy, and extensive versatility. At the same time, the effectiveness and security of gene editing primarily depend on the transmission of CRISPR elements into specific cells. This assessment outlines and contrasts key delivery techniques such as microinjection, electroporation, hydrodynamic injection, membrane deformation, sonoporation, lance array nanoinjection (LAN), and lipid nanoparticles (LNPs). Electroporation and LAN demonstrate significant efficiency and accuracy in embryonic and cellular systems, whereas LNPs and sonoporation provide low-toxicity, clinically applicable platforms for in vivo uses. Hydrodynamic injections are especially efficient for delivering to the liver, while membrane deformation facilitates editing in difficult to transfect cells. Every method offers distinct advantages, and the choice should be customized to the particular therapeutic or research setting. Ongoing advancements in CRISPR delivery mechanisms are crucial to maximize its capabilities in precision medicine and gene therapy.

Keywords: CRISPR-Cas9, Genome alteration, Methods of delivery, Electroporation, ipid Nanoparticles, Nano-injection, Sonoporation, Hydrodynamic injection, Genetic engineering.

INTRODUCTION:

In recent times, CRISPR-related (Cas) endonucleases such as Cas9 have become effective therapeutic instruments for addressing genetic diseases. Initially identified as a bacterial defense system against viruses, Cas9 has been modified to cleave DNA in an RNA-directed fashion in different cell types. The Cas9 protein creates an active ribonucleoprotein (RNP) complex with a guide RNA (gRNA) that guides it to a precise 20-nucleotide DNA sequence. This sequence pairs with the host genome, and aided by a protospacer-adjacent motif (PAM), allows Cas9 to create a double-strand break (DSB) at the targeted location.[1] Genome editing methods, including those utilizing chemical or ultraviolet (UV) mutagenesis, DNA recombinase-mediated gene substitution, zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), have greatly enhanced both basic biology and clinical studies.

A newer and groundbreaking advancement is the CRISPR/Cas system, which originates from the adaptive immune response of the bacterium Streptococcus pyogenes. This technology has transformed genome editing, broadening its use among many species like Drosophila, Caenorhabditis elegans, zebrafish, mice, rats, silkworms, and humans[2]. CRISPR/Cas9 has notably become a prominent genome editing technique due to its accuracy and longevity, frequently needing just one intervention to produce enduring results. The CRISPR/Cas9 mechanism consists of the Cas9 endonuclease and guide RNAs usually designed as a single-guide RNA (sgRNA) to guide the Cas9 protein to the specific DNA location[3].

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and related Cas proteins create an adaptive immune defense in bacteria and archaea, safeguarding against foreign genetic materials such as plasmids and viruses. Found in 1987 and designated in 2002, CRISPR's function in immune defense was proposed in 2005 when scientists identified spacer sequences that corresponded with viral and plasmid DNA. Cas9, directed by a single-guide RNA (sgRNA), can accurately slice DNA, rendering CRISPR-Cas9 a powerful and adaptable gene-editing instrument. It is commonly utilized in fundamental research, biotechnology, agriculture, and medicine[5].CRISPR/Cas9 is notable for its exceptional efficiency and accuracy. Directed by RNA, Cas9 identifies and cleaves particular DNA sequences, facilitating gene deletions or insertions using a DNA repair template.

CRISPR/Cas9 delivery techniques resemble other nucleic acid transfection methods and consist of viral vectors, chemical vectors, and physical approaches. Mechanical or electrical forces are employed in physical transfection to temporarily disrupt cell membranes, enhancing the ingestion of editing components. Progress in micro- and nanotechnologies has resulted in new physical delivery methods such as nanostructure-mediated electroporation, enhancing transfection efficiency and cell viability by offering consistent treatment in contrast to conventional bulk electroporation[6].

How does CRISPR-Cas9 work?

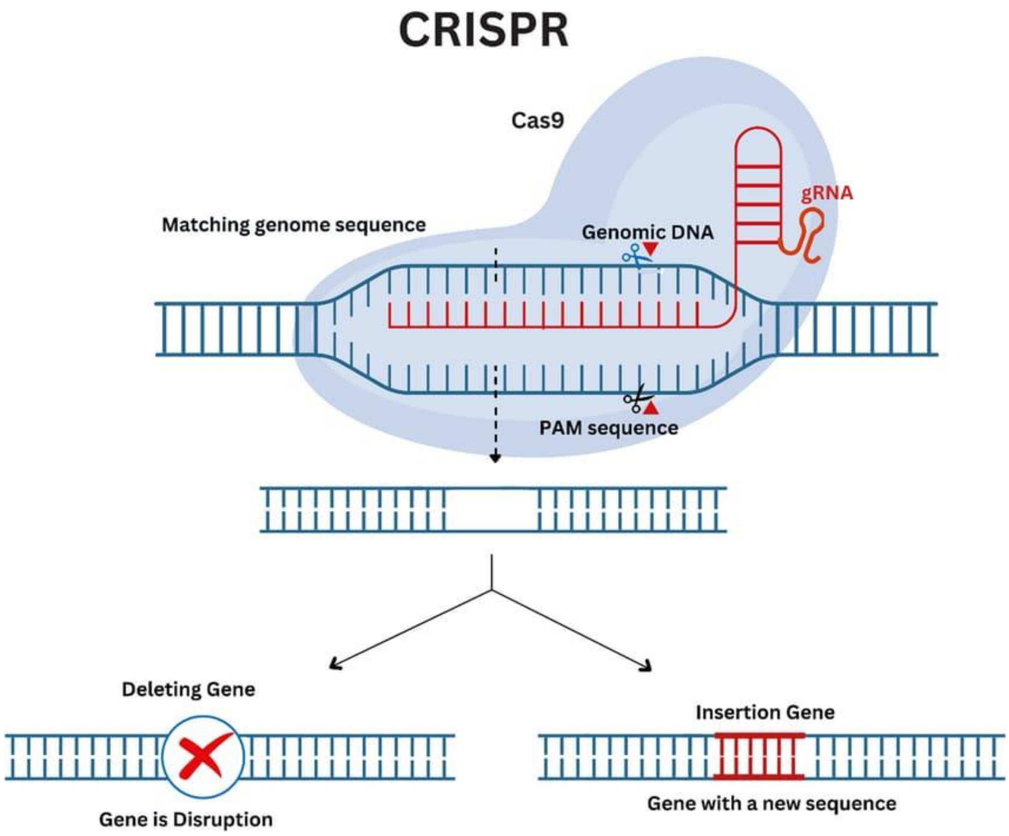

Figure1. **Mechanism of CRISPR Cas9**

The CRISPR-Cas9 system comprises two essential components that create a modification (mutation) in the DNA. These include:

a) A protein known as **Cas9**
   - This functions like a pair of 'molecular scissors' that can snip the two strands of DNA at a precise site in the genome, allowing segments of DNA to be inserted or eliminated.

b) A segment of RNA known as **guide RNA (gRNA)**.
   - This comprises a short segment of pre-designed RNA sequence (approximately 20 bases in length) situated within a longer RNA framework. The scaffold region attaches to DNA, and the predetermined sequence directs Cas9 to the correct section of the genome. This ensures that the Cas9 enzyme cleaves at the correct location in the genome.
   - The guide RNA is created to locate and attach to a particular sequence in the DNA. The guide RNA contains RNA bases that are complementary to the target DNA sequence found in the genome. This indicates that, theoretically, the guide RNA is expected to attach solely to the target sequence and not to any other parts of the genome.
   - The Cas9 enzyme tracks the guide RNA to the identical site in the DNA sequence and creates a cut through both strands of the DNA. At this point, the cell identifies the DNA as being damaged and attempts to fix it.

MATERIALS AND METHODS:

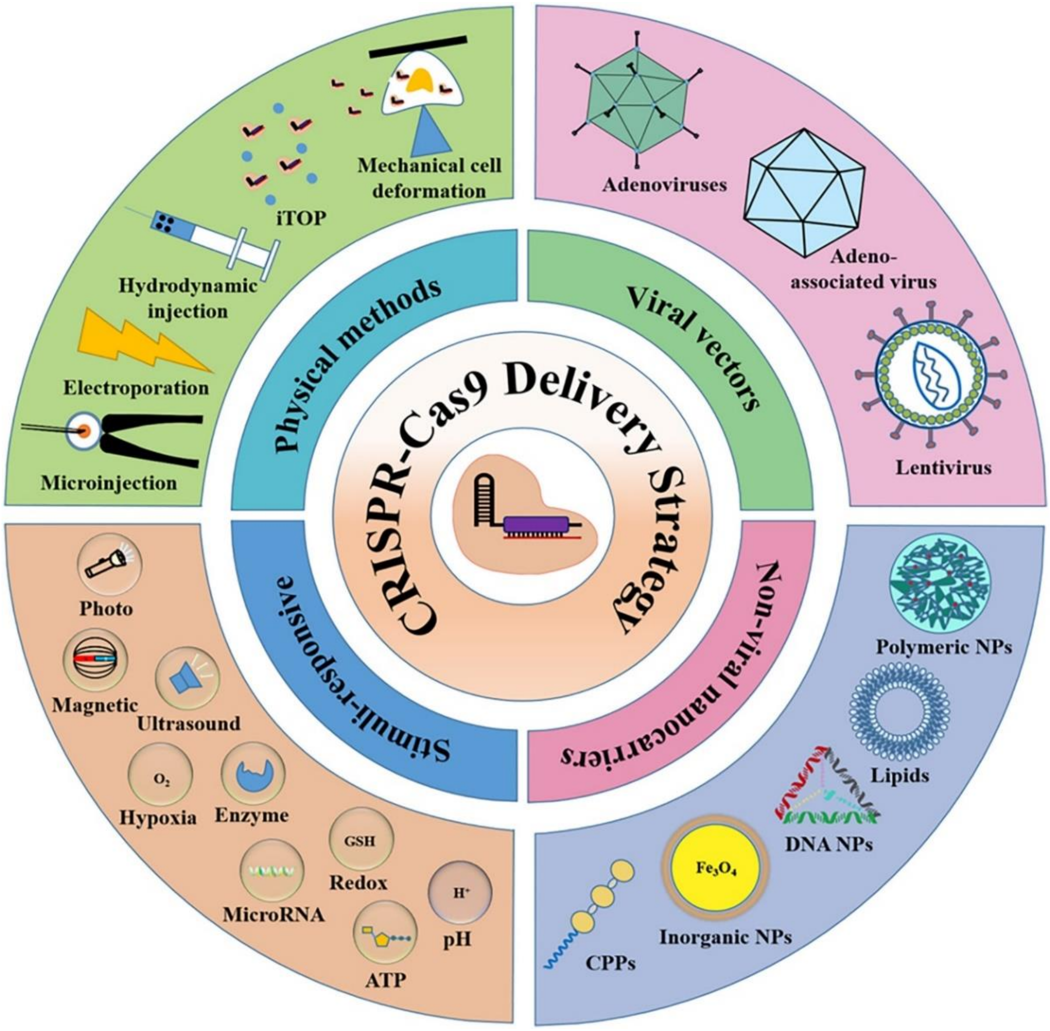

Figure 2. CRISPR-CAS9 Delivery System

Microinjection

Microinjection with CRISPR/Cas9 includes creating a combination of Cas9 protein, guide RNA (sgRNA), and optional donor DNA within an injection buffer. The solution is injected into fertilized embryos with thin glass needles while being observed under a microscope. After injection, embryos are incubated and analyzed for genome modifications through PCR or sequencing[8].

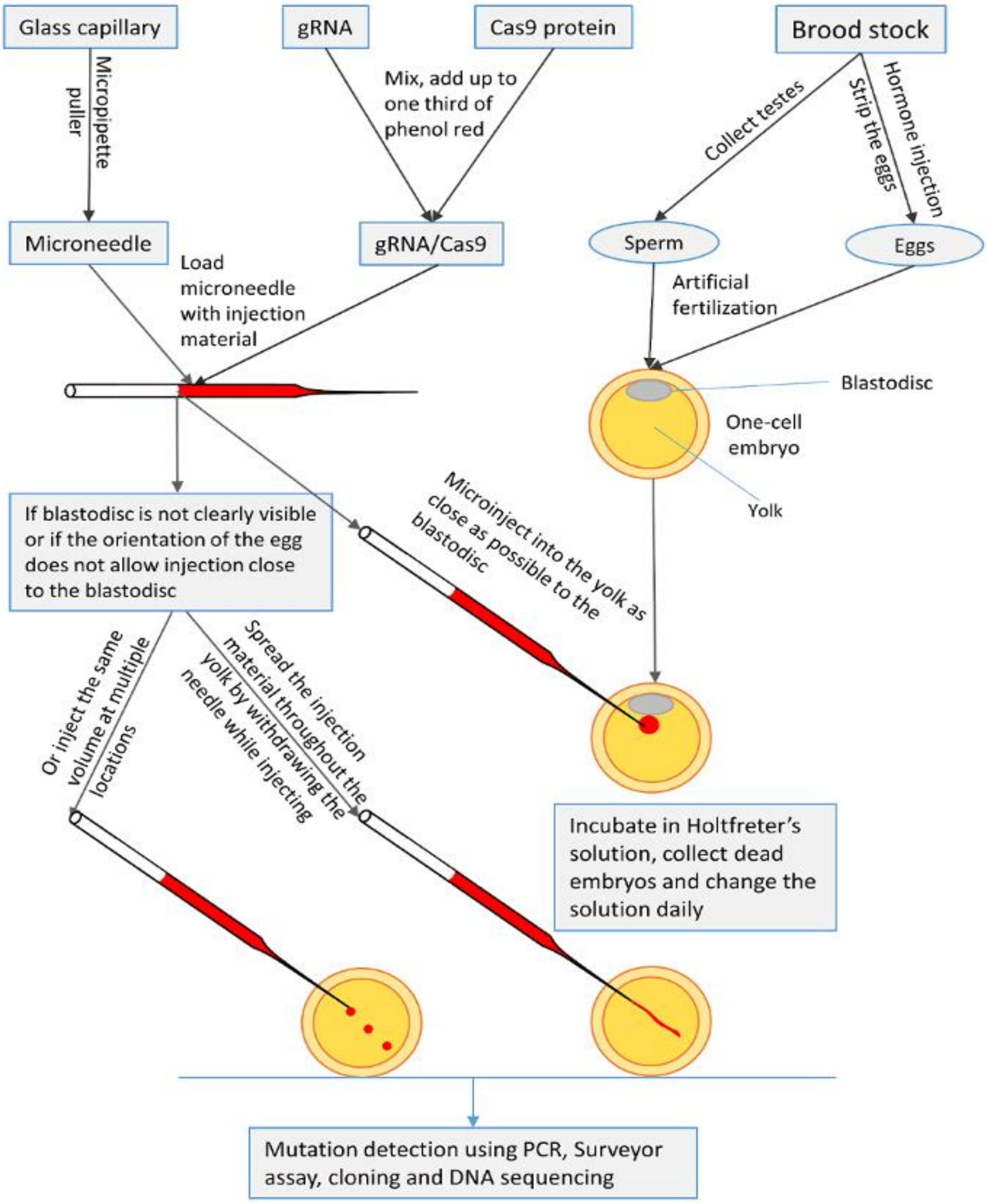

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Figure 3.Microinjection of CRISPR/Cas9 Protein into Embryos for Gene Editing

Electroporation

Cells are readied and combined with CRISPR-Cas9 elements (Cas9 protein or mRNA, gRNA, and optional repair templates). The solution is electroporated with optimized settings specific to the cell type and method of delivery. After electroporation, cells are allowed to recover before analyzing gene editing through PCR, sequencing, or additional assays[7].

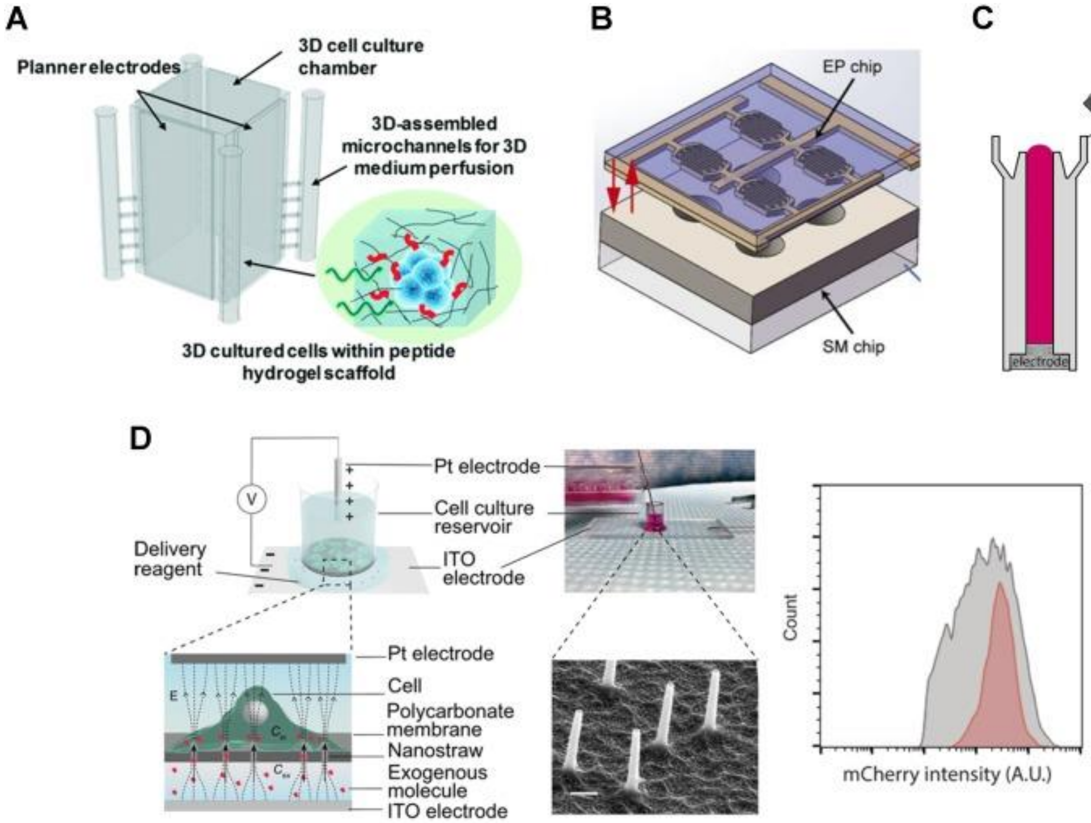

Figure 4.Novel electroporation platform for transfection and Schematic of 3D microfluidic electroporation system

1.1. Hydrodynamic injection

CRISPR/Cas9 elements (plasmids or RNPs) are dissolved in sterile saline and swiftly injected into the tail vein of the animal using a syringe. The injection volume (for instance, 8–10% of body weight in mice) allows for systemic distribution, particularly to the liver[9].

1.2. Deformation of the membrane

Microfluidic devices exert regulated mechanical forces on cells, facilitating the efficient introduction of CRISPR/Cas9 elements. Cas9 and gRNA whether alone or in RNPs are delivered into different cell types, including difficult-to-transfect ones such as stem and lymphoma cells[10].

1.3. Sonoporation

Ultrasound-assisted delivery employs CRISPR-Cas9 elements (DNA plasmids or RNPs) paired with microbubbles featuring a gas core and lipid/protein shell. Ultrasound devices produce sound energy to temporarily make cell membranes permeable, facilitating CRISPR absorption. This technique is utilized on cultured cells or tissues for gene modification[12].

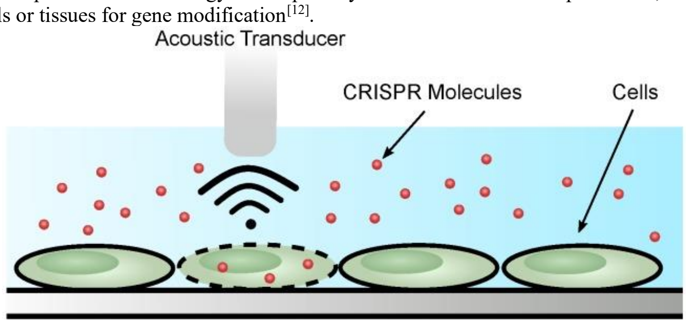

Figure 5.Acoustoporation methods for cell transfection

1.4. Lance array nanoinjection

Lance Array Nanoinjection employs a silicon chip featuring tiny lances covered in CRISPR-Cas9 elements to breach cell membranes. An electrical current aids in introducing Cas9 and sgRNA into cultured cells, with parameters such as current strength and cycle count being fine-tuned. After injection, the efficiency of editing is evaluated through PCR, sequencing, or fluorescence based methods[13] .

1.5. Lipid nanoparticle systems

Lipid nanoparticles (LNPs) were created with SpCas9 protein and sgRNA to produce RNP complexes for gene editing, employing specific lipid ratios and buffers such as HEPES or PBS. The LNPs were analyzed for their size, charge, and stability, and evaluated in HEK293T cells through fluorescence assays, T7E1 digestion, and flow cytometry to determine editing efficiency[1].

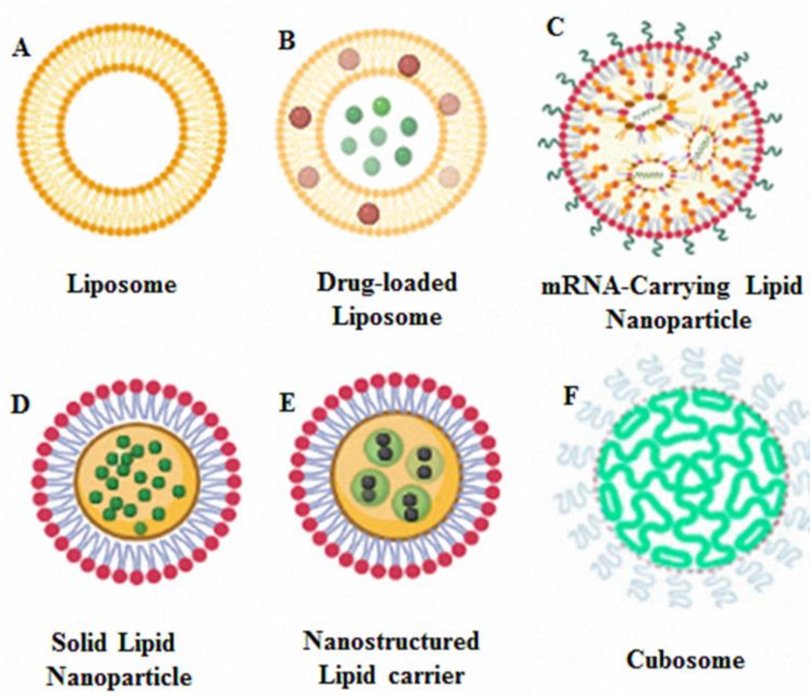

Figure 6. Schematic representation of A - liposome, B - Drug-loaded liposome encapsulating hydrophobic and hydrophilic drugs, C - mRNA-Carrying lipid nanoparticle, D - Solid lipid nanoparticle, E - Nanostructured lipid carrier, and F - Cubosome

RESULT AND DISCUSSION:

Table 1. Results and highlights of different delivery methods

Delivery Method	Key Results	Discussion Highlights
Electroporation (ZEN)	➢ 57% alteration at 600/300 ng/ml ➢ Cas9/sgRNAHDR: 27% (Tet2 region) ➢ No off-targets found	➢ Effective, safe zygote modification ➢ Elevated birth rates versus microinjection ➢ Expandable and adaptable to stress
Microinjection	➢ FokI-dCas9:≥50% alterations inembryos ➢ Success in multiplex editing (Reg3b/Reg3g) ➢ 10% integration of plasmid	➢ Exact, high-accuracy modification ➢ Challenging from a technical standpoint ➢ Beneficial for inbred and cryopreserved oocytes
Hydrodynamic Injection	➢ Approximately 40% transfection of hepatocytes ➢ Prolonged gene activity ➢ Targeting specific to the liver	➢ Basic, non-viral liver transfer ➢ Beneficial for studies involving inducible and zonal expression
Membrane Deformation	➢ >90% EGFP knockout (MDA-MB-231) ➢ Wide-ranging applicability across cell types ➢ Success in endogenous gene editing	➢ Efficient, viable distribution at scale ➢ Perfect for cells that are difficult to transfect ➢ Not viral and able to scale
Nonoperation	➢ 78.2% of editing is done with the US ➢ In vivo tumor reduction ➢ 58.77% NFE2L2 knockout ➢ Few unintended targets	➢ Merges CRISPR with sonodynamic treatment ➢ Safe, highly effective, and specific to tumors
LAN (Nanoinjection)	➢ As much as 93.8% GFP loss (4.5 mA, 3×) ➢ Numerous injections significantly improved efficiency	➢ Extremely effective non-viral technique ➢ Adjustable, low toxicity ➢ Better than viral vectors in HeLa cells
Lipid Nanoparticles	➢ Approximately 47.4% transfection ➢ Approximately 16.1% gene modification (PLK-1) ➢ 67% decrease in tumor volume	➢ Transferring sizable plasmids efficiently ➢ Reduced toxicity, superior to Lipofectamine ➢ Moderate adjustments, intense impacts

COMPARATIVE CONCLUSION:

Among the different CRISPR-Cas9 delivery techniques investigated, lance array nanoinjection (LAN) proves to be the most beneficial for non-viral, high-efficiency gene editing. It reached a knockout efficiency of up to 93.8% with low cytotoxicity, outperforming viral techniques in HeLa cells. Electroporation (ZEN) is extremely efficient for embryo editing, providing scalability, elevated birth rates, and accurate HDR-based alterations, rendering it perfect for creating animal models. Lipid nanoparticles (LNPs) offer a low-toxicity, clinically applicable method for in vivo delivery of large plasmids, albeit with moderate editing effectiveness. Sonoporation uniquely merges gene editing with targeted tumor therapy, delivering high editing efficiency and therapeutic outcomes while ensuring exceptional biosafety. Hydrodynamic injections continue to be useful for liver-focused research, whereas membrane deformation is more effective for challenging-to-transfect cells invitro.

Similarly, the selection of technique relies on the specific application: LAN is best for editing high-efficiency cell lines, ZEN for zygotic and germline modifications, LNPs for delivering systemic therapies, and sonoporation for gene therapy aimed at cancer. In general, LAN and ZEN stand out as the most adaptable and effective methods with regard to editing accuracy, delivery effectiveness, and safety.

CONCLUSION:

The CRISPR-Cas9 technology has transformed gene editing due to its ease of use, accuracy, and adaptability. At the same time, its therapeutic effectiveness is heavily reliant on effective and secure delivery techniques. Every assessed technique extending from conventional microinjection and electroporation to sophisticated methods such as lipid nanoparticles, sonoporation, and lance array nano injection provides distinct advantages tailored to particular uses. For example, electroporation and LAN achieve high editing efficiency in embryos and cultured cells, whereas LNPs and sonoporation present encouraging in-vivo delivery methods with low toxicity. Hydrodynamic injections continue to be an important method for therapies aimed at the liver. In the end, choosing a delivery method must correspond with the specific cell type, required efficiency, scalability, and safety in clinical settings, making ongoing innovation in CRISPR delivery systems crucial for the progress of gene therapy and precision medicine. The success of CRISPR-Cas9 gene editing largely depends on improved delivery methods. Every approach offers distinct benefits includes electroporation and LAN provide great efficacy in embryos and cells, LNPs and sonoporation demonstrate potential in clinical applications for targeted tumor or systemic delivery and hydrodynamic injections continue to be optimal for gene therapy in the liver.

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