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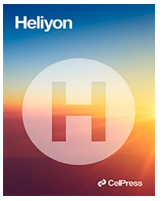

Review article

Lipid nanoparticles: The game-changer in CRISPR-Cas9 genome editing

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ABSTRACT

The steady progress in genome editing, especially genome editing based on the use of clustered regularly interspaced short palindromic repeats (CRISPR) and programmable nucleases to make precise modifications to genetic material, has provided enormous opportunities to advance biomedical research and promote human health. However, limited transfection efficiency of CRISPR-Cas9 poses a substantial challenge, hindering its wide adoption for genetic modification. Recent advancements in nanoparticle technology, specifically lipid nanoparticles (LNPs), offer promising opportunities for targeted drug delivery. LNPs are becoming popular as a means of delivering therapeutics, including those based on nucleic acids and mRNA. Notably, certain LNPs, such as Polyethylene glycol-phospholipid-modified cationic lipid nanoparticles and solid lipid nanoparticles, exhibit remarkable potential for efficient CRISPR-Cas9 delivery as a gene editing instrument. This review will introduce the molecular mechanisms and diverse applications of the CRISPR/Cas9 gene editing system, current strategies for delivering CRISPR/Cas9-based tools, the advantage of LNPs for CRISPR-Cas9 delivery, an overview of strategies for overcoming off-target genome editing, and approaches for improving genome targeting and tissue targeting. We will also highlight current developments and recent clinical trials for the delivery of CRISPR/Cas9. Finally, future directions for overcoming the limitations and adaptation of this technology for clinical trials will be discussed.

1. Background

One of the most vital methods for elucidating relationships between various biological phenotypes and genetic barriers is through gene editing. The CRISPR (clustered regularly interspaced short palindromic repeats) system, originally developed as a programmable endonuclease for inducing DNA double-strand breaks (DSBs), has evolved into a programmable endonuclease particle. Moreover, specific earlier gene-editing techniques, such as Lambda-red enhanced recombination and step-by-step allelic displacement, have found application in the manipulation of bacterial genomes [1]. Meganucleases (MNs), Zinc Finger Nucleases (ZNFs), Transcription

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Activator-Like Effector Nucleases (TALENs), and CRISPR-Cas9 are among the gene-editing techniques that hold promise for simplifying clinical applications. CRISPR-Cas9 is currently the most extensively utilized gene-editing technique in biomedicine due to its low cost, ease of use, high efficiency, and rapid adaptability. CRISPR, along with the CRISPR-associated (Cas) protein—an intracellular protein [2]—originates from prokaryotes (bacteria and archaea). These prokaryotes retain memories of past viral infections as a defense mechanism to prevent re-infection [3]. They achieve precise DNA targeting by forming complementary base pairs with the target DNA using a single guide RNA (sgRNA) and subsequently cleaving the DNA at specific sites [4].

Among the three types of CRISPR mechanisms identified (type I, type II, and typw III), type II is the most efficient. In the type II mechanism stores DNA of previously encountered phages in its CRISPR locus between short palindromic repeats (~20 base pairs), which can be transcribed into CRISPR RNA that guides the Cas9 nuclease to cut and inactivate the invading bacteriophage. Synthetic versions of CRISPR RNA directs the Cas9 nuclease to produce site-specific DNA double-stranded breaks (DSBs) [5]. The remarkable success of the CRISPR-Cas9 genome editing approach stems from the simplicity of generating guide RNA sequences that target Cas9. This targeting drives the nuclease to specific DNA loci, ensuring both efficiency and specificity [6]. From targeting dominant genes responsible for cancer to modifying genomes underlying genetic diseases, the applications of CRISPR-Cas9 in healthcare are boundless [2].

The remarkable advancement in nanotechnology has significantly influenced the fields of biomedicine and drug delivery. This progress has led to the development of new theraputic approuch, leveraging nanocarriers or nanovectors to precisely deliver active pharmaceutical ingredients (API) to specific locations at the right concentration and time. These nano-sized units, when combined with polymeric matrices, provide a more controlled release profile, offering potential benefits for treatment [7]. Nanoparticles have become essential in biological research due to their structural and dimensional resemblance to biological molecules. Their extensive surface area and volume make them effective antimicrobial agents, exhibiting strong antibacterial properties. This enables their use in various applications such as diagnostics, cell labeling, biomarkers, drug delivery, cancer therapy, and water purification [8]. Researchers have explored the utilization of Lipid Nanoparticles (LNPs) for delivering CRISPR-Cas9 systems to diverse cell types, both for therapeutic purposes and the development of knockout animal models. Nanoparticles can be modified to alter their genetic components thanks to their precise targeting, scalability, modification efficiency, low potential for immune response, and reduced susceptibility to nucleases [9]. Additionally, their ease of manipulation, self-assembly capability, biocompatibility, high bioavailability, payload capacity, and diverse physicochemical properties have made LNPs the most prevalent class of FDA-approved nanomedicines [10]. Cationic ionizable lipids play a pivotal role in LNPs by facilitating efficient encapsulation of nucleic acids, cellular transport, and endosomal release. The lipid layer additionally shields nucleic acids from degradation [11]. This protective layer safeguards CRISPR components from RNases, enzymatic breakdown, and immune responses while aiding their cellular entry [10]. Despite these advantages, LNP systems can be limited by low drug loading and biodistribution, leading to significant accumulation in the liver and spleen [9]. This review explores the various benefits of LNPs in CRISPR-Cas9 delivery systems, resulting in therapeutic advancements and the utilization of this technology for genome manipulation. In this review paper, the databases such as Science Direct, PubMed and Scopus, Emerald and Google Scholar search engine were searched for valid articles using combination of keywords such as "CRISPR-Cas9, Drug delivery, Genome editing, Lipid nanoparticles". This systamtic review were performed from December 2022, to November 2023. After reviewing the papers, 150 relevant articles were selected, and after careful examination 109 papers were approved at the final stage.

2. Mechanisms of CRISPR-Cas 9 for genome editing

Genome editing is a form of genetic engineering that entails the insertion, deletion, or modification of DNA within living cells [12]. The term CRISPR refers to short, partially repetitive DNA sequences present in prokaryotic genomes. Prokaryotes utilize CRISPR, along with its associated proteins [13], as part of their defense mechanism against viruses and bacteriophages [14]. The CRISPR-Cas9 system consists of three major components: A) a single-guide RNA (sgRNA) crRNA array, which comprises a 20-nucleotide sequence that binds to the target DNA; and B) the *trans*-activating crRNA (tracrRNA), a short noncoding RNA that forms a functional guide RNA by base-pairing with the crRNA (gRNA). PAM (Protospacer Adjacent Motif) is a Cas9 nuclease that cleaves the DNA strand three to four bases after the protospacer neighboring motif. The PAM is a short, conserved sequence motif (2–5 bp) located on the invading DNA, playing a pivotal role in the selection and cleavage of the target DNA. It is positioned adjacent to the crRNA-targeted sequence [15]. The Cas9 nuclease possesses two essential domains: the RuvC-like domain and the HNH domain, each responsible for cleaving one DNA strand. The tracrRNA region of the guide functions as a handle for Cas9, with the crRNA spacer sequence guiding the complex to specific infectious sequences [16]. There are a total of six distinct forms of CRISPR-Cas systems found in a wide range of bacteria and archaea, categorized into Class 1 (types I, III, IV) and Class 2 (types II, V, VI). Class 1 systems involve multiple Cas proteins working together, whereas Class 2 systems feature a single Cas protein, making them more user-friendly and efficient for genome editing. CRISPR-Cas9 Type II, one of the most renowned and extensively studied of the Class 2 systems, is particularly well-known [17]. As a result of the immune response to invading genetic material, small segments of foreign DNA are integrated into the host genome during the immunization process [18]. As a consequence, a host can defend against the same invader due to the genetic history left by a prior infection [19]. Subsequently, the CRISPR array undergoes transcription and enzymatic processing to generate short, mature CRISPR RNAs (crRNAs). During the silencing process, tracrRNA aligns with the repeating segment of crRNA, creating a distinctive dual RNA hybrid structure, akin to a jigsaw puzzle piece [20]. This dual-RNA guide directs the Cas9 scissor protein to cleave any DNA possessing a complementary 20-nucleotide (nt) target sequence and an adjacent PAM sequence, following the binding of tracrRNA to crRNA [21]. Cas9 executes the cleavage of double-stranded DNA through its two nuclease domains: HNH and RuvC, ultimately resulting in a double-strand break (DSB). The HNH-like nuclease domain cleaves the DNA strand complementary to the guide RNA sequence (target

strand). The RuvC-like nuclease domain cleaves the DNA strand opposite the complementary strand (non-target strand) [22]. Cas9, a large DNA endonuclease discovered in S. pyogenes, exhibits multiple domains and functions. Mutations in one of the two nuclease domains result in the cleavage of only one strand of DNA. Specifically, when the RuvC catalytic domain is mutated, rendering it inactive, the HNH catalytic domain exclusively cuts the complementary strand to the single-guide RNA (sgRNA) [21]. This variant is known as Nickase Cas9 or nCas9. When both nCas9s are applied to adjacent Protospacer Adjacent Motifs (PAMs) at the target locus, a double-strand break (DSB) occurs, significantly enhancing specificity. Cas9 induces a DSB, which is subsequently repaired by two error-prone pathways [23]. Homology-directed repair (HDR) and non-homologous end joining (NHEJ) are two DNA repair mechanisms [24]. NHEJ often leads to small, random insertions and deletions (InDel) at the cleavage site, causing DNA frame-shifts and/or premature stop codons, ultimately resulting in gene knockout. Conversely, the HDR pathway allows for the precise replacement of a defective or mutated sequence with the wild-type sequence, resulting in accurate genome alterations. To initiate HDR, a donor DNA template is utilized to introduce the correct DNA sequence at the desired location. In summary, while HDR offers a more reliable DNA repair pathway, it is generally less efficient than the NHEJ mechanism (24). Targeted genome editing, in contrast to random mutagenesis methods such as EMS and radiation, enables precise and effective modifications at specific genomic sites. The CRISPR-Cas9 system achieves sequence specificity through the unique structure and conformation of the Cas9 protein. Cas9 is a bi-lobed protein consisting of a conserved core and two nucleic acid binding grooves: a large recognition (REC) lobe and a small nuclease (NUC) lobe, connected by a helical bridge [25,26]. The NUC lobe houses two nuclease domains, RuvC and HNH, as well as a PAM-interacting domain (PI), which dictates Cas9's specific function. In its natural state, Cas9 is inactive. However, when it interacts with sgRNA at its REC lobe, it becomes activated. The Cas9-sgRNA complex scans the double-stranded DNA for target sites with the appropriate protospacer adjacent motif (PAM) via Watson–Crick base pairing between the sgRNA and the targeted DNA, typically characterized by the trinucleotide NGG. When connected to the relevant protospacer adjacent motifs (PAMs), the HNH nuclease domain cleaves the RNA–DNA hybrid, while the RuvC domain cleaves the complementary strand, resulting in a double-strand break (DSB). Both

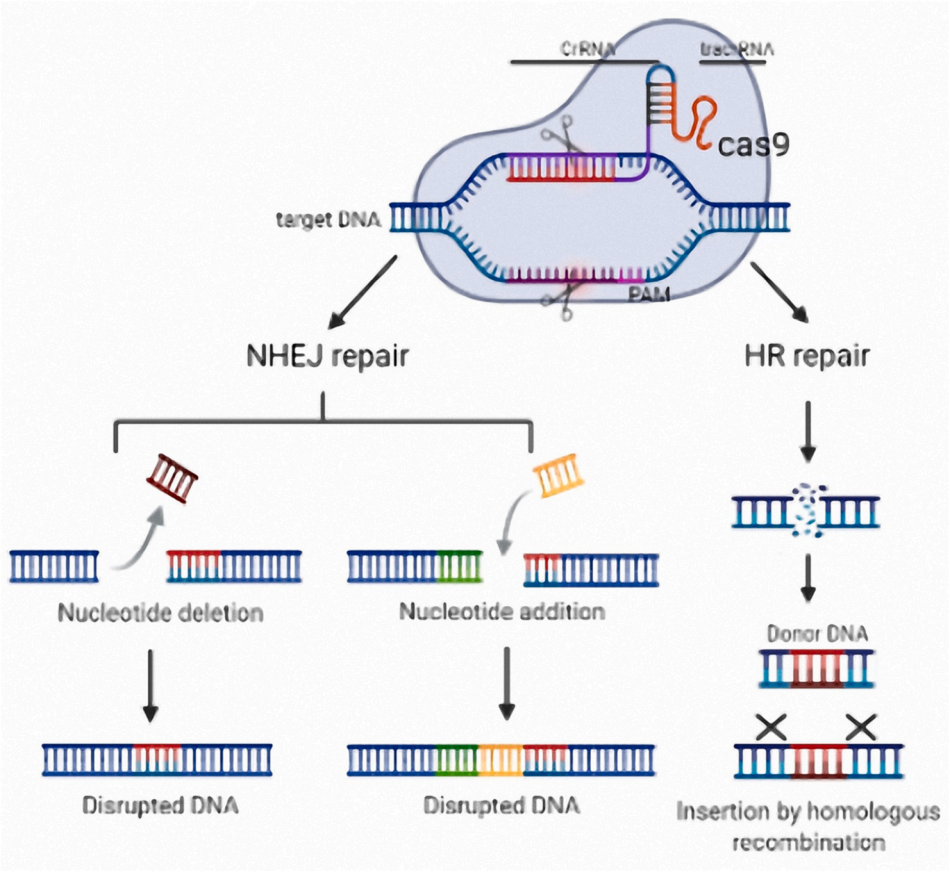

Fig. 1. CRISPR-Cas9 is a strong genome editing technique. The protospacer is recognized by the Cas9 complex when it is combined with sgRNA. Only if this sequence is followed by a Protospacer Adjacent Motif (PAM) is this conceivable. In order to modify genomic sequences, the tracrRNA hybridizes with the crRNA and attaches to the Cas9 protein, generating the CRISPR–Cas9/sgRNA complex. The Cas9–sgRNA complex unwinds dsDNA, and sgRNA's complementary sequence anneals to one of the DNA strands. Endonuclease domains break both DNA strands three bases upstream of the PAM sequence when they bind. If an adequate donor is present, a double-strand break (DSB) in DNA forms, which is subsequently repaired either by a homology directed repair (HDR) pathway or by non-homologous end joining (NHEJ). NHEJ introduces short insertions, deletions, or indels, while HDR repair enables precise genome editing at the target site.

prokaryotes and eukaryotes possess endogenous non-homologous end joining (NHEJ) and homology-directed repair (HDR) systems capable of repairing DSBs [27]. NHEJ employs DNA ligase IV to rejoin the broken ends, often leading to insertions or deletions (Indels), while HDR utilizes a homologous complementary template to repair DSBs, typically resulting in error-free repairs. NHEJ is advantageous for gene knockout applications, whereas HDR is commonly used in plants for gene replacement and gene knock-in Ref. [28]. The CRISPR-Cas9 system has surpassed previous genome editing technologies, such as zinc finger nucleases (ZFNs) and engineered transcription activator-like effector nucleases (TALENs), both of which rely on the nuclease domain of *Fok*I endonucleases to create double-strand DNA breaks [29]. CRISPR-Cas9 stands out for its ease of modification compared to ZFN and TALENs, thus offering broader potential applications. For instance, ZFN is composed of a series of Cys2–His2 ZF domains, each binding to a different protospacer adjacent motif (PAM), which makes it challenging to select the correct target sequence. When two ZFNs cooperate to recognize a unique 18–24 bp DNA sequence, they form a dimer. However, the adoption of ZFN and TALEN technologies is limited due to concerns about off-target effects, the complexity of developing modular DNA-binding proteins, and context-dependent binding requirements [30] (see Fig. 1).

3. Applications of CRISPR-Cas9

In just a few years after its discovery, the CRISPR-Cas9 genome editing system has been investigated for a wide variety of applications and has had a significant impact on various fields, including health, agriculture, and biotechnology. Researchers anticipate further advancements in this technology that could lead to the treatment and cure of diseases, the development of more nutritious crops, and the eradication of infectious diseases [31]. Below are some of the latest applications and clinical observations related to CRISPR-Cas9 currently under evaluation.

3.1. Role in gene therapy

So far, nearly 6000 genetic diseases have been identified, but the majority of them lack viable therapeutic options [32]. Gene therapy, which involves replacing a faulty gene with foreign DNA and editing the mutant gene at its natural site, represents the latest advancement in the field of medical biotechnology. From 1998 to August 2019, 22 gene therapies were licensed for the treatment of human disorders, including the groundbreaking CRISPR-Cas9 technology [33].

Since its discovery in 2012, CRISPR-Cas9 gene editing has held the potential to treat a wide range of known recessive genetic diseases, including sickle cell disease, thalassemia, cystic fibrosis, and muscular dystrophy [34]. CRISPR-Cas9 has been applied in clinical studies aimed at treating sickle cell disease (SCD) and thalassemia [35]. SCD is an autosomal recessive genetic disorder affecting red blood cells, caused by a point mutation in the β-globin chain of hemoglobin, resulting in the formation of sickle hemoglobin (HbS). The polymerization of HbS during deoxygenation leads to severe clinical consequences, including hemolytic anemia [36]. CRISPR-Cas9 has also been explored as a potential therapy for cystic fibrosis. Cystic fibrosis results from a genetic mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which impairs the structural stability and function of the CFTR protein [37]. The CFTR protein, regulated by protein kinase-A, is located on the apical surface of epithelial cells in various tissues, including the lungs, intestines, pancreas, and reproductive tract, and functions as an anion channel protein. Current therapies for cystic fibrosis primarily focus on symptom management, including the use of antibiotics, bronchodilators, and mucus-thinning medications, along with CFTR-modifying pharmaceuticals, which aim to alleviate symptoms and reduce associated risks, although a cure remains elusive [38]. Researchers are actively exploring gene-editing technologies and molecular targets to address this genetic condition. Among these technologies, CRISPR-Cas9 holds significant promise, despite being in its early developmental stages. In 2013, researchers successfully corrected the mutation at the CFTR locus in intestinal stem cells from two cystic fibrosis patients, resulting in the production of the correct gene and the restoration of full protein activity. Recent demonstrations have highlighted the potential of CRISPR-Cas9 in treating cystic fibrosis. Additionally, Duchenne muscular dystrophy (DMD), characterized by muscle weakness and caused by a mutation in the dystrophin gene, has seen successful repair in patient-induced pluripotent stem cells using CRISPR-Cas9. Despite considerable efforts, the primary therapeutic approach for DMD remains supportive rather than curative. To restore dystrophin expression in DMD-affected muscles, multiple therapeutic techniques, including gene therapy, cell therapy, and exon skipping, are currently under investigation [39]. Novel and effective strategies for repairing the DMD gene include CRISPR-Cas9-mediated deletion/excision of intragenic DNA and removal of the duplicated exon, which facilitates the restoration of dystrophin protein production [40]. "The first human study of a CRISPR-based treatment was conducted to address resistant lung cancer. T-cells were extracted from the blood of three patients and subsequently modified in the laboratory using CRISPR-Cas9 technology to delete genes (TRAC, TRBC, and PD-1) that could impede the immune system's ability to target cancer cells effectively. These engineered T-cells were then reintroduced into the patients. Engineered T-cells have the potential to specifically target antigens and eliminate cancer cells. Remarkably, no adverse side effects were reported, and the modified T-cells could still be detected approximately ten months after the procedure [41].

The gene-editing technique CRISPR-Cas9 holds the potential to be used in the treatment of infectious diseases caused by microorganisms. Researchers are particularly focused on addressing HIV, the immunodeficiency virus. In May 2017, a team of researchers from Temple University demonstrated in animal models that the excision of the HIV-1 genome using CRISPR-Cas9 could completely halt HIV-1 replication and eliminate the virus from infected cells [42].

CRISPR-Cas9 technology may also be employed to prevent HIV from entering host cells by editing the chemokine co-receptor type-5 (CCR5) genes in these cells, in addition to targeting the HIV genome. For instance, a Chinese in vitro study revealed that CRISPR-Cas9 genome editing of CCR5 caused no cell toxicity or infection, and the researchers concluded that the modified cells were more effective

at preventing HIV infection than unmodified cells [43].

3.2. Role in the generation of animal models

Gene targeting, relying on homologous recombination and embryonic stem cells, has long been the standard method for modifying animal genomes. This approach has played a crucial role in establishing a causal relationship between genomic mutations and developmental phenotypes, as well as diseases. However, in certain organisms, the application of gene targeting is limited due to its time-consuming techniques and the absence of embryonic stem cells. Recent studies have demonstrated that CRISPR-Cas9 technology is capable of rapidly generating targeted genomic alterations in the germ lines of various model species [44], significantly advancing functional genomics. Effective in vivo gene modification in one-cell stage zebrafish embryos can be achieved through the microinjection of Cas9-encoding mRNA and adaptable sgRNA, offering a simplified, rapid, and cost-effective method [45]. In mouse zygotes, co-injection of Cas9 mRNA and sgRNAs targeting different genes has resulted in the generation of mutant mice with biallelic mutations, demonstrating the high efficiency of CRISPR/Cas-mediated gene editing for disrupting multiple genes simultaneously. Furthermore, co-injection of Cas9 mRNA/sgRNAs with mutant oligos has led to the creation of gene knock-in mice with precise point mutations in two genes. Other studies have shown that reporter and conditional mutant mice can be generated in a single step by co-injecting mouse zygotes with Cas9 mRNA, different sgRNAs, and DNA vectors of varying sizes. Additionally, mice with desired deletions have been created using sgRNAs targeting two distinct sites in a gene [46]. Multiplex activation of endogenous genes can be achieved by injecting a two-component transcriptional activator consisting of a nuclease-dead Cas9 protein combined with a transcriptional activation domain and sgRNAs targeting gene promoters [47].

4. LNP as a novel gene delivery technique for theraputic application

LNPs are a recently developed form of drug formulation that encases biological molecules like nucleic acids (DNA, and RNA) and

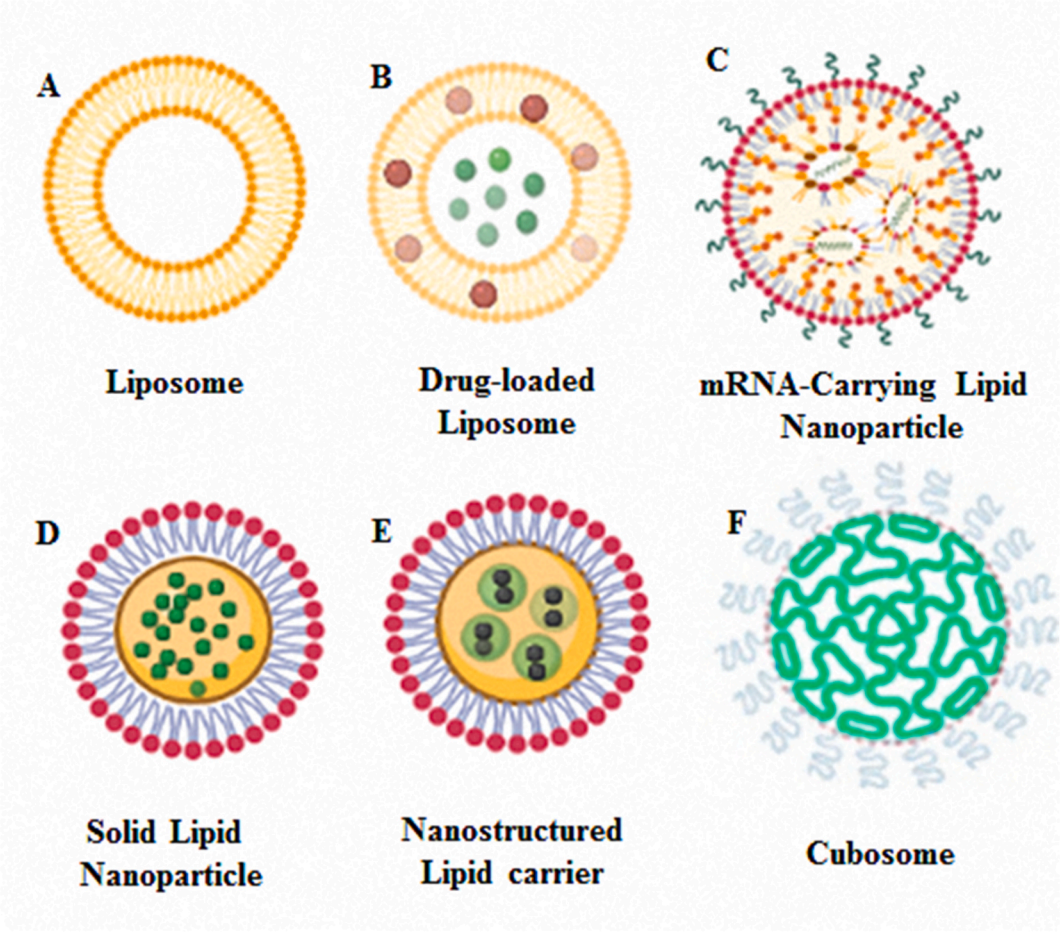

Fig. 2. Schematic representation of **A.** liposome, **B.** Drug-loaded liposome encapsulating hydrophobic and hydrophilic drugs, **C.** mRNA-Carrying lipid nanoparticle, **D.** Solid lipid nanoparticle, **E.** Nanostructured lipid carrier, and F. Cubosome

proteins, as well as a combination of both. LNPs are spherical vesicles that can be visualized using electron microscopy. In the context of therapeutic applications, LNPs are typically less than 100 nm in diameter and are composed of lipids and nucleic acid payloads. The concept of liposomes, which are sealed lipid bilayer vesicles (Fig. 2A), originated in the 1960s when it was discovered that they form spontaneously in water. Liposomes are simpler lipid vesicles composed of phospholipids and cholesterol. They are larger in size compared to LNPs. LNPs are a more recent development that evolved from the concept of liposomes [48,49]. In the early 1990s, the term "lipid nanoparticle" was coined, marking the onset of the era of nanoscience and nanotechnology. Liposomes have been utilized for research purposes, particularly in the study of the physical chemistry of lipids in aqueous environments. Additionally, they have been explored for potential clinical applications. To prepare liposomes, lipids are typically dried using a rotary evaporator. Subsequently, they are suspended within an aqueous solution and subjected to sonication. This process generates multi-lamellar vesicles, which appear as a milky suspension [48]. Their potential as drug delivery systems was recognized shortly after their discovery. Given that more than 40 % of small-molecule cancer drugs exhibit poor water solubility, the need for drug delivery mechanisms capable of encapsulating these compounds and enhancing their aqueous solubility became evident. Liposomes were the pioneering nanomedicine delivery technology to successfully transition from concept to clinical application, with several approved pharmaceutical formulations. These versatile drug delivery platforms have the ability to encapsulate hydrophilic drugs within their aqueous core and hydrophobic

Table 1
Clinical Trials of LNP-Formulated mRNA Drugs and Vaccines against diseases.

Disease	Biological active/Encoding Sequence	Clinical trial phase	Clinical trial identifier	Reference
CMV	mRNA-1647/Pentamer complex and full- length membrane-bound glycoprotein B and pp65 T cell antigen of CMV	Phase II	NCT04232280, NCT03382405	[68]
CMV	mRNA-1443/Pentamer complex and full- length membrane-bound glycoprotein B and pp65 T cell antigen of CMV	Phase I	NCT03382405	[68]
Zika	mRNA-1893/Structural proteins of Zika virus	Phase I	NCT04064905	[69]
Zika	mRNA-1325/Structural proteins of Zika virus	Phase I	NCT03014089	[69]
hMPV/PIV3	mRNA-1653: Fusion proteins of hMPV and PIV3	Phase I	NCT04144348, NCT03392389	[70]
Influenza A (H10N8)	mRNA-1440 (VAL-506440)/Influenza Hemagglutinin H10N8 (A/JiangxiDonghu)	Phase I	NCT03076385	[70]
RSV	mRNA-1345/Presumed to be F protein	Phase I	NCT04528719	[70]
RSV	mRNA-1777 (V171)/Presumed to be F protein	Phase I	Unregistered	[70]
Rabies	CV7202/Rabies virus G protein	Phase I	NCT03713086	[71]
Chikungunya	mRNA-1388 (VAL-181388)/"Viral antigenic proteins" Program appears to have been replaced by mRNA encoding a monoclonal antibody	Phase I	NCT03325075	[72]
COVID-19	ChulaCov19 mRNA/SARS-Cov2-spike protein-binding IgG antibody	Phase I/II	NCT04566276	[71]
COVID-19	self-amplifying mRNA (SAM) platform/anti-Spike IgG antibodies GMCs	Phase I	NCT04758962	[71]
COVID-19	mRNA-1273/Full-length, prefusion-stabilized spike protein	Phase I	NCT04283461	[71]
COVID-19	mRNA-1273/Full-length, prefusion-stabilized spike protein	Phase III	NCT04811664	[71]
Melanoma	mRNA-4157/personalized cancer vaccine targeting 20 tumor- associated antigens	Phase II	NCT03897881	[73]
Melanoma	RBL001.1; RBL002.2; RBL003.1; RBL004.1/malignant melanoma-associated antigens	Phase I	NCT02410733	[73]
Melanoma, Colon cancer, Gastrointestinal cancer, Genitourinary cancer, hepatocellular cancer	NCI-4650/mRNA-based, Personalized Cancer Vaccine	Phase I/II	NCT03480152	[74]
Melanoma, NSCLC, Bladder Cancer, Colorectal Cancer, Triple Negative Breast Cancer, Renal Cancer, Head	RO7198457/personalized cancer vaccine targeting tumor- associated antigens	Phase I	NCT03289962	[74]
Cancer- Solid Tumor, Lymphoma, Ovarian	mRNA-2416/mRNA-2416/OX40L, a T-cell co-stimulator, IL-23 and IL-36γ pro-inflammatory cytokines	Phase I and II	NCT03323398	[75]
Solid tumors	mRNA-4157/personalized cancer vaccine targeting 20 tumor- associated antigens	Phase I	NCT03313778	[75]
Cancer- various	mRNA-2752/Human OX40L, IL-23, and IL-36γ	Phase I	NCT03739931	[76]
Ovarian Cancer	W_ova1 vaccine: Three ovarian cancer tumor associated antigens mRNAs	Phase I	NCT04163094	[77]
Adult Glioblastoma	Autologous total tumor mRNA and pp65 full length lysosomal associated membrane protein (105) mRNA loaded DOTAP liposome vaccine	Phase I	NCT04573140	[78]
Hepatocellular Carcinoma	PLK1 (polo-like kinase-1)	Phase I and II	NCT01262235	[79]

CMV, cytomegalovirus; hMPV, human metapneumovirus; RSV, respiratory syncytial virus.

drugs within the lipid bilayer's hydrocarbon chain region (Fig. 2B). Among the first anticancer nanoformulations and liposomal medications to gain approval were Doxil and Myocet, both based on doxorubicin [50]. Doxil, for instance, was developed to enhance the circulation of doxorubicin in human plasma and reduce cardiotoxicity by utilizing the enhanced permeability and retention (EPR) effect with stabilized nanoparticles (>100 nm). It was designed for intravenous injection in the treatment of advanced ovarian cancer, multiple myeloma, and HIV-related Kaposi's sarcoma [51,52]. Doxil consists of hydrogenated soy phosphatidylcholine, cholesterol, and DSPE-PEG2000 LNPs [53]. AmBisome (amphotericin B), DaunoXome (daunorubicin), DepoCyt (cytarabine), DepoDur (morphine), and Visudyne (verteporfin) are among the approved liposomal medications for clinical use [54]. However, until recently, despite clinical approval, no FDA-approved liposomal medications demonstrated a significant improvement in overall survival (OS) compared to their parent treatments. A notable breakthrough occurred in a 2017 study, where Phase III results for liposomal combination drugs, such as cytarabine–daunorubicin (Vyxeos; CPX-351), were compared to their individual counterparts cytarabine and daunorubicin ("7 + 3"), in patients aged 60 to 75 with high-risk acute myeloid leukemia. This study revealed a superior OS of 9.56 months compared to 5.95 months in the 60 to 75-year-old patient group [55]. Epaxal, a protein antigen formulation in LNPs for hepatitis vaccination, is another liposomal medication [56]. Several amphotericin B nanoparticle formulations based on lipids have been developed, demonstrating favorable pharmacokinetic profiles and significantly reduced side effects [57]. Utilizing LNP carriers (Fig. 2C) represents one of the most effective strategies for delivering nucleic acid medications [58] (Fig. 2).

Nucleic acids are delivered to cells through both viral and nonviral vectors. Among nonviral methods for delivering therapeutic nucleic acids, cationic LNPs are the most commonly utilized. These LNPs are stable complexes composed of synthetic cationic lipids and anionic nucleic acids [59]. A wide range of cationic lipid amphiphiles has been developed and assessed for their suitability as carriers for nucleic acids. Ionizable lipids are preferred over nonionizable cationic lipids due to their lower potential for causing harm. The release of nucleic acids from their complexes with cationic lipids is a crucial step in nucleic acid delivery once they have entered the cell. Anionic lipids within the cell likely contribute to the release of nucleic acids from LNPs by neutralizing the charge of their cationic lipid carriers and breaking the electrostatic bonds between them and the nucleic acids. The interaction between anionic lipids and cationic lipids can alter nanoparticle architecture, leading to nonlamellar formations [60]. It has been hypothesized that the ability of cationic lipid vectors to induce the development of nonlamellar lipid phases correlates with their effectiveness in delivering nucleic acids [61]. In 1989, DOTMA, a cationic lipid, and its synthetic derivative, DOTAP, became the first lipids used to deliver mRNA [62]. Their positively charged amines facilitate the encapsulation of negatively charged RNA. To reduce the production of transthyretin protein in the liver, the FDA recently approved patisiran (Onpattro), a siRNA packaged in LNPs. This marks not only the first approved siRNA medicine but also the first LNP-formulated nucleic acid drug, representing a significant advancement in nucleic acid therapies [63]. A noteworthy recent application of LNPs is in the development of COVID-19 messenger RNA (mRNA) vaccines by Pfizer/BioNTech and Moderna. These vaccines were rapidly designed and have shown exceptional efficacy in disease prevention [64,65]. The SARS-CoV-2 spike protein, which triggers an immune response against the virus, is produced from the mRNA delivered into the cytoplasm of host cells by these vaccines [66].

Disease-fighting mRNA drugs and vaccines have shown promise, with several candidates advancing to human clinical trials (Table 1). LNPs enable the intracellular transport of mRNA, allowing for the synthesis of almost any required protein within host cells [67]. Nucleoside-modified mRNA vaccines targeting Zika virus, cytomegalovirus, tuberculosis, and influenza, as well as LNP-based mRNA vaccines for the same diseases, have all entered clinical trials (see Table 1). The use of mRNA therapeutic vaccines in cancer immunotherapy, particularly against melanoma, ovarian cancer, breast cancer, and other solid tumors, holds significant potential (Table 1). In 2016, the first study involving LNP-formulated mRNA for protein replacement therapy was reported. This study focused on LNP-encapsulated mRNA encoding human frataxin as a potential therapeutic agent for Friedreich's ataxia [67].

While liposomes have proven valuable as drug carriers, they involve complex production procedures using organic solvents, exhibit limited drug entrapment efficiency, and present challenges for scaling up. To address some of these issues, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) were developed (see Fig. 2D and E). Unlike conventional liposomes, which consist of liquid-crystalline lipid bilayers, SLNs are composed of solid lipids, while NLCs consist of a blend of solid and liquid-crystalline lipids [80,81]. The particle sizes of both SLNs and NLCs typically range from 40 to 1000 nm. SLNs and NLCs offer improved physical stability, addressing a major concern associated with liposomal formulations. They are also easier to manufacture on a larger scale without the need for organic solvents, exhibit higher loading capacities and cargo bioavailabilities compared to other LNPs, and demonstrate greater stability during sterilization. One of the earliest examples of a drug delivery composite system combining solid lipid nanoparticles (SLN) and hydrogels was achieved using methacrylate-modified dextran (dextran-MA) [82]. In this system, the hydrogel was rapidly formed through UV photoactivation of the dextran methacrylate moieties, effectively encapsulating the pre-dispersed SLN within the polymeric network in the aqueous solution. Importantly, the structure of the SLNs remained intact throughout the hydrogel formation process. To assess drug release behavior, ibuprofen was encapsulated within the SLNs. Subsequent experiments revealed that only 40 % of the encapsulated ibuprofen was released after a 2-h incubation in a 0.1 M HCl solution, simulating gastric transit. Several systems, including ibuprofen-dextran-MA and ibuprofen-loaded SLN, exhibited burst release characteristics. In a study conducted by Paolicelli et al. the pharmaceutical efficacy of the SLN-dextran system was investigated at various pH levels. Remarkably, at both pH 5 and pH 7, the entire loaded SLN-dextran system demonstrated comparable effectiveness to the commercial cream (Nizoral), highlighting its ability to preserve the action of the encapsulated molecule [83]. SLNs and NLCs hold promise not only in gene therapy but also in the treatment of ocular diseases, infectious diseases, and lysosomal storage disorders. Particularly, topical administration of antifungals such as clotrimazole and ketoconazole has shown significant success when utilizing SLNs and NLCs. Ocular drug delivery represents a crucial method, yet it holds vast untapped potential. Due to the absence of inflammatory lipid content in SLNs, they may be considered an ideal choice for ocular drug delivery. The use of nonlamellar LNPs in drug delivery has also been a subject of study [84]. In the 1980s, the first reports of nonlamellar lipid phases, including inverted cubic and hexagonal liquid-crystalline phases,

emerged in controlled release formulations for inhaled medication administration [85]. Cubosomes, which possess a significantly larger membrane surface area compared to liposomes, are suitable for loading membrane proteins and small-molecule medicines (Fig. 2F). These unique characteristics make cubosomes applicable in various fields, including drug delivery systems, membrane bioreactors, artificial cells, and biosensors [86]. Cervin et al. conducted the first in vivo investigation using cubosomes for taxane delivery. Docetaxel was loaded at a concentration of 5 wt% relative to lipids in a PC-diolein-based formulation. This formulation featured particles with a diameter of 80–90 nm and demonstrated stability for up to one year when stored at 2–8 °C. In mouse models carrying PC3 cell-inoculated human prostate cancer xenografts, intravenous injection of the LCNP formulation led to a more significant reduction in relative tumor volume compared to treatment with the reference commercial product Taxotere. The LCNP formulation also exhibited improved excipient tolerance, attributed to the presence of endogenous PC components and a lower proportion of polysorbate [87]. Wu et al. conducted a study involving cubosomes designed for the nasal administration of a peptide drug in an Alzheimer's disease rat model. These cubosomes were functionalized with OL (MW 1.7 kDa) to enhance mucoadhesiveness by specifically binding OL to L-fucose. In a biodistribution analysis employing fluorescent 6-coumarin, OL-coated cubosomes demonstrated a threefold higher accumulation in brain tissue compared to their uncoated counterparts [88].

The development of LNPs, including various types such as nano-structured lipid carriers and ionizable cationic nanoparticles, has significantly enhanced their drug delivery capabilities. These advancements have expanded the application possibilities of LNP formulations. LNPs have demonstrated remarkable potential in genetic medicine, where their ability to efficiently deliver nucleic acids into cells is crucial for gene editing, vaccine production, and other genetic therapeutics [89].

5. LNP delivery of CRISPR/Cas9 genome-editing

The power of gene editing lies in its ability to precisely target and modify the genetic material within living cells. This approach holds immense promise for addressing the root causes of genetic diseases, offering the potential to prevent or treat a wide range of hereditary and acquired disorders. By utilizing gene editing tools, such as CRISPR-Cas9, scientists can make precise changes to the DNA sequence, correcting genetic defects or introducing beneficial genetic modifications. This approach has the potential to revolutionize the treatment of genetic diseases, as it offers the possibility of permanent and targeted intervention. However, the direct injection of nucleic acids, such as plasmid DNA or mRNA encoding Cas9, for in vivo gene editing encounters several significant challenges include (1) hydrophobicity; nucleic acids are inherently hydrophobic, meaning they repel water and are poorly soluble in aqueous environments. This property makes it difficult for nucleic acids to cross the cell membrane and enter cells efficiently; (2) negative Charge: nucleic acids carry a strong negative charge due to the phosphate groups in their backbone. This negative charge can hinder their cellular uptake, as the cell membrane is also negatively charged; (3) Instability: Nucleic acids are susceptible to degradation by enzymes present in the body, such as nucleases. This instability limits their circulation time in the bloodstream and reduces their ability to reach target cells; (4) Immunogenicity: Nucleic acids can trigger an immune response when introduced into the body. This immune response can lead to the production of antibodies that neutralize the nucleic acids and prevent them from reaching their intended

Table 2
Summary of Studies LNPs to Deliver CRISPR/Cas9 in the forms of mRNA, Ribonucleoprotein (RNP) and Plasmid DNA (pDNA).

LNP System	CRISPR/Cas9 cargo	Advantages	Limitations	ref
Lipid Nonoparticles (LNP)	Chemically modified sgRNA/Cas9 mRNA and sgRNA/Cas9 RNP	Low immunogenicity, high biocompatibility Low toxicity, very simple for large-scale manufacture CRISPR-off-target Cas9's effects might be decreased by a temporary release.	Endosomal degradation of cargo, Specific cell tropism	[13, 92, 93]
LNP System/liposomes/ lipoplexes	Cas9 mRNA sgRNA/RNP	Virus-free, Simple manipulation, Low cost	Endosomal degradation of cargo, Specific cell tropism	[94]
Ionizable LNPs	Cas9 mRNA and co-delivered sgRNA targeting PCSK9 (for selective organ targeting (SORT)	High endosomal escape, Biodegradable, Cumulative gene editing upon repeated dosing in vivo	N/A	[94]
Ionizable LNPs	Cas9 mRNA and co-delivered sgRNA targeting DMD1	Restoration of gene expression	Hepatotoxicity increase in plasma	[95]
NTLA-2002 biodegradable LNP	Cas9 mRNA and co-delivered an sgRNA targeting targeting KLKB1	Tissue-specific delivery	N/A	[96]
Cationic arginine functionalized Gold Nonoparticles	sgRNA/Cas9 glut (+NLS) RNP targeting AAVS1 gene (or PTEN gene)	FDA-approved, Low stress to the cells	Variable efficiency depends on cell types, Requires extensive optimization	[97]
LNP−pDNA delivery containing DLin-KC2-DMA and unsaturated PCs	pDNA targeting EGFP	N/A	Moderate cellular toxicity	[98]

Pcsk9 = proprotein convertase subtilisin/kexin type 9, SORT = selective organ targeting, DMD1 = dystrophin gene, NLS = nuclear localization signal, DLin-KC2-DMA = 2,2-dilinoleyl-4-(2-dimethylaminoethyl)- [1,3]-dioxolane A, PC = phosphatidylcholine, EGFP = enhanced green fluorescent protein, NA= Not reported.

targets, and (5) Off-target Effects: gene editing approaches that utilize nucleic acids, such as CRISPR-Cas9, can potentially lead to off-target effects, where the Cas9 enzyme cuts DNA at unintended locations in the genome. This can result in unintended mutations and genomic instability [90]. To overcome these challenges, various strategies have been developed, including the use of delivery systems such as viral vectors, LNPs, and cell-penetrating peptides. These delivery systems can protect nucleic acids from degradation, enhance their cellular uptake, and reduce off-target effects. A significant benefit of employing LNPs as drug carriers is their capability to evade detection by the innate immune system and prolong their circulation time. These characteristics are particularly valuable for delivering hydrophobic drugs with short circulation half-lives, such as nucleic acids and proteins. Prolonged circulation time allows LNPs (including those with CRISPR components in either nucleic acid or protein formats) to effectively reach target tissues and facilitate precise therapeutic genome editing [91].

LNPs can deliver CRISPR components in various formats. The typical methods include encapsulating (1) plasmid DNA (pDNA) encoding both Cas9 and gRNA, (2) Cas9 mRNA and gRNA simultaneously or separately, and (3) Cas9/sgRNA (protein/RNA) RNP complex. Each of these techniques has its own advantages and limitations, requiring distinct LNP-specific formulation criteria to ensure optimal compatibility without sacrificing functionality. Commercially available transfections reagents originally designed for delivery of plasmids and siRNAs can be used to deliver plasmid-based Cas9/gRNA, RNA mixtures of Cas9 and sgRNA, and even RNPs to cell lines such as HEK293FT, U2OS, mouse ESCs, N2A, and A549. However, in vivo applications of these transfection reagents, including lipofectamine, are limited by their cytotoxic and inflammatory effects. The development of novel synthetic ionizable cationic lipids and LNP formulations has overcome many of these barriers and has made LNP-mediated therapeutic gene editing a realistic prospect [92]. The ability of wide variety of LNP-Mediated delivery systems for CRISPR-Cas9 genome editing has been independently confirmed (see Table 2), and are further discussed below [90] (Fig. 3).

As menitoes above, the CRISPR/Cas9 gene-editing system can be delivered using pDNA forms of both Cas9 and gRNA. Kulkarni et al. utilized an LNP formulation that incorporated DLin-MC3-DMA and substituted the saturated helper lipid distearoylphosphatidylcholine (DSPC) with unsaturated phosphatidylcholine (PC) helper lipids to enhance LNP-pDNA delivery. Furthermore, the efficacy of formulations combining different ionizable cationic lipids with unsaturated PC helper lipids was evaluated. The most efficient formulation included DLin-KC2-DMA and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) in primary embryonic mesenchymal cells obtained from chicken embryos. In contrast to lipofectamine, which achieved only 50 % transfection and 33 % cell viability, this formulation resulted in over 85 % cell viability and over 90 % transfection efficiency [99]. In another study, PEG-lipid/AuNPs/Cas9-sgPlk-1 (LACP), thermally-triggered nanoparticles, were employed for the administration of the CRISPR-Cas9 system. The inner core of LACP consisted of Cas9-sgPlk-1 and TAT peptide-modified AuNPs, while the outer layer was composed of lipids including DOTAP, DOPE, cholesterol, and PEG2000-DSPE. Upon laser irradiation, the Cas9-sgPlk-1 plasmid could be released into the cytosol from the gold core, and the TAT peptide could facilitate the plasmid's entry into the nuclei to target gene knockout. Lipid encapsulation in the outer shell of nanoparticles can enhance their stability. The results showed that LACP-mediated melanoma suppression was significant both in vitro and in vivo [100]. In other study, a Polyethylene glycol-phospholipid-modified cationic lipid nanoparticle (PLNP) was designed as a core–shell cationic liposome for efficient CRISPR-Cas9 plasmid delivery, both in vitro and in vivo [11]. To create the core, the Cas9/sgRNA plasmid was combined with protamine-chondroitin sulfate conjugation, which acted as a condensation aid for the plasmid. Positively charged lipid membranes composed of 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), dioleoylphosphatidylthanolamine (DOPE), and cholesterol, were then applied to the core. These lipid membranes were further modified with 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-amino (polyethylene glycol) (DSPE-PEG) to create stable PLNP. The target gene for the study was PLK1, a mitosis regulator. In the A375 cell line, PLNP/DNA demonstrated a transfection efficiency of 47.4 % and an indel mutation rate of 16.1 %, both significantly higher than those achieved with Lipo2000. Additionally, in melanoma-bearing mice, the in vivo transfection efficiency was assessed. Tumor growth was inhibited by approximately 67 % when

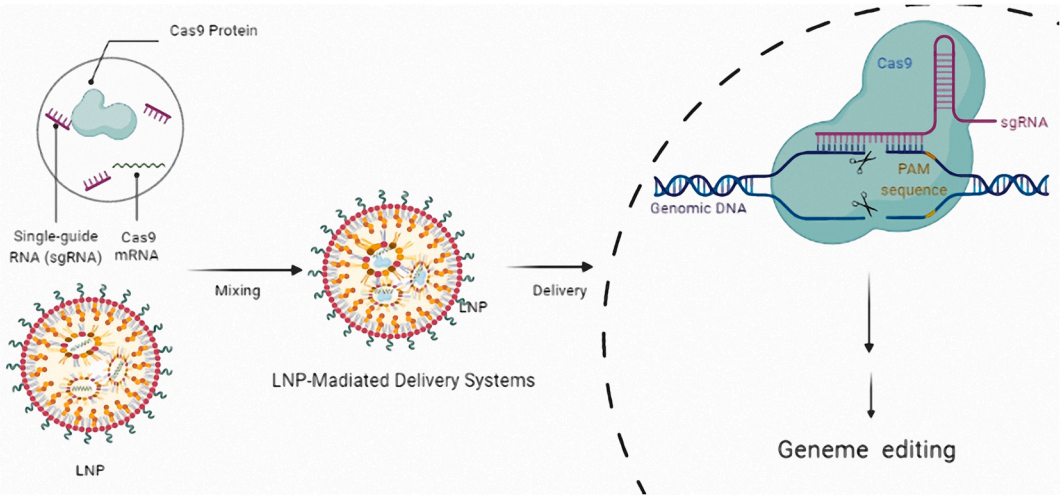

Fig. 3. LNP-Mediated delivery systems for CRISPR-Cas9 genome editing.

compared to the PBS-treated group. Furthermore, it outperformed PLNP/siPLK-1 in terms of reducing tumor volume, achieving a 50 % decrease. Therefore, PLNP was considered a suitable gene carrier for cancer treatment [100]. Morever, modifying nanoparticles with specific ligands enhances the cell-specific uptake of CRISPR-Cas9-loaded nanocarriers, further promoting receptor-mediated cellular uptake of ligand-conjugated nanocarriers. For instance, Zhang et al. employed TAT peptides-conjugated gold nano-clusters modified with 4-aminophenyl-D-galactopyranoside (Gal-PEG-DSPE) instead of DSPE-PEG to achieve efficient hepatocyte-targeted delivery of Cas9/sgPCSK9. The inclusion of Gal in Gal-LGCP facilitates hepatocytic absorption through the asialoglycoprotein receptor (ASGPR)-mediated pathway. In vivo studies demonstrated that Gal-LGCP effectively downregulated PCSK9 expression in hepatocytes, leading to a reduction of approximately 30 % in serum LDLC levels [101]. Together, these strategies improved encapsulation efficiency, resultant cellular uptake, CRISPR/Cas9 expression, and ultimately genome editing efficiency.

Instead of using pDNA delivery, there is a growing focus on delivering Cas9 mRNA in both in vitro and in vivo settings. LNPs can be utilized to encapsulate both Cas9 mRNA and gRNA in the same particles, or to encapsulate them separately. Finn et al. have investigated the ability to modify the molecular structure of Cas9 gRNA to enhance Cas9 editing efficiency in vivo and to target the Ttr gene, a monogenic over-expressed target in several rare amyloidosis disorders [102]. In another study, Qiu et al. developed a non-viral LNP-mediated CRISPR-Cas9 delivery system for the distribution of Cas9 mRNA in the liver, demonstrating its efficacy by targeting the Angptl3 gene. They utilized bioreducible LNPs for Cas9 mRNA and gRNA delivery, displaying highly effective in vitro genome editing as well as rapid reduction of the Pcsk9 cholesterol-regulating gene in vivo, highlighting the effectiveness of this delivery approach. The system comprises a leading tail-branched bioreducible lipidoid (306-O12B) co-formulated with an optimal combination of excipient lipid molecules, efficiently co-delivering SpCas9 mRNA and a sgRNA targeting Angptl3 (sgAngptl3) in a single dose. 306-O12B LNP successfully delivered Cas9 mRNA and sgAngptl3 to the liver hepatocytes of wild-type C57BL/6 mice, resulting in a median editing rate of 38.5% and a 65.2% reduction in serum ANGPTL3 protein levels. Moreover, the knockdown of Angptl3 in the liver led to a significant decrease in LDL-C and TG levels. Importantly, there were no signs of off-target mutagenesis or liver toxicity at the nine highest-predicted sites. The therapeutic level of CRISPR-mediated genome editing was maintained for at least 100 days following a single dose [103].

Precisely optimizing LNPs for tissue-specific gene-editing tool delivery is crucial. LNPs represent a diverse group of molecules capable of transporting therapeutic nucleic acids to various tissues, exemplified by Onpattro, a short interfering RNA LNP treatment for transthyretin-mediated amyloidosis recently approved by the US Food and Drug Administration (FDA). Cheng et al. have demonstrated that rational design involving different lipid formulations or compositions enables tissue-specific delivery of the CRISPR-Cas9 tool. SORT LNPs designed for targeting the lungs, spleen, and liver were developed to edit therapeutically relevant cell types, including epithelial cells, endothelial cells, B cells, T cells, and hepatocytes. SORT LNPs exhibit compatibility with various gene-editing techniques, including mRNA, Cas9 mRNA/single guide RNA, and Cas9 ribonucleoprotein complexes, particularly within specific tissues. This compatibility proves beneficial in advancing the development of treatments for protein replacement and gene correction. To illustrate SORT LNPs' potential for editing therapeutically important targets on a significant scale, they co-delivered Cas9 mRNA and an sgRNA targeting PCSK9, a highly promising target for familial hypercholesterolemia and atherosclerotic cardiovascular disease. The results revealed that 20 % of DODAP SORT LNPs effectively induced indels at the PCSK9 locus, with 60 % of these mutations being validated by TIDE analysis. This led to a 60 % mutation rate in the PCSK9 sequence and a 100 % reduction in PCSK9 serum levels. This study provides a foundational framework for the rational enhancement of existing nano-vectors for organ-specific targeting in delivery [104].

Using CRISPR RNPs is expected to reduce off-target effects, improve editing efficiency, and increase target specificity by up to 10 times compared to plasmid-based CRISPR/Cas9 DNA or mRNA delivery methods. As a result, extensive efforts have been made to enhance LNP formulations for the efficient encapsulation and delivery of CRISPR RNPs to specific targets. In a study by Wang et al. they developed bioreducible lipid nanoparticles that can efficiently transport polyanionic RNPs to target GFP-expressing HEK cells and help them escape from endosomes. The study demonstrates that these bioreducible lipids can deliver proteins into cells, facilitate their release from endosomes in response to the intracellular environment, and guide them to their target sites within the cell. This study also showed that this delivery system is effective for genome editing in cultured human cells, as well as in the mouse brain for in vivo gene recombination [105]. Similary, Zuris et al. have discovered a method using common cationic lipid nucleic acid transfection reagents to efficiently deliver polyanionic protein into human cells, even in the presence of serum. This approach has successfully delivered various proteins, including Cre recombinase, TALE- and Cas9-based transcription activators, and Cas9:sgRNA nuclease complexes. In experiments, unmodified Cas9:sgRNA complexes achieved up to 80 % genome editing with higher specificity compared to DNA transfection. Furthermore, this method has shown successful delivery of RNPs proteins into the mouse inner ear in vivo, achieving high levels of recombination and genome modification in hair cells [106]. In other study, Wei et al. developed a method to create and engineer modified lipid nanoparticles that efficiently deliver RNPs into cells and affect various tissues, including muscle, brain, liver, and lungs. Intravenous infusion enabled tissue-specific, multiplexed editing of six genes in mouse lungs. High carrier potency was utilized to create organ-specific cancer models in the livers and lungs of mice by simply deleting several genes. The RNPs were also capable of restoring dystrophin expression in DMD mice and significantly reducing PCSK9 levels in both serum and liver tissue of C57BL/6 mice using the developed carriers. 5A2-DOT-10 LNPs, which encapsulated Cas9/sgDMD RNPs, were injected weekly into the Tibialis Anterior (TA) muscles of DMD exon 44 deletion mice to restore dystrophin expression. The Western blot results were quantitated, demonstrating a 4.2% recovery of dystrophin protein. In the 5A2-DOT-5 LNPs encapsulating Cas9/sgPCSK9 RNPs treated group, T7EI assay results confirmed indel creation at the PCSK9 gene loci [98].

LNPs, which are recognized for their natural tendency to target and gather in the liver, offer a hopeful delivery approach for treating genetic disease. The efficiency, expandability, and temporary nature of LNP-based delivery for CRISPR systems make it an appealing choice for clinical application (Table 2). In November 2020, Intellia Therapeutics initiated the first clinical trial using LNPs

as a CRISPR/Cas9 delivery vector (NCT04601051) [107]. Hereditary transthyretin amyloidosis with polyneuropathy, specifically ATTRv-PN, is targeted for treatment with NTLA-2001, a CRISPR/Cas9 gene therapy administered intravenously. This therapy aims to modify the TTR gene in hepatocytes (refer to Table 1 for details). The interim trial data published in 2021 included six individuals with ATTRv-PN. These participants were divided into two groups, with half of them receiving a high dosage (0.3 mg/kg) of NTLA-2001, while the other half received a low dose (0.1 mg/kg). After the first infusion, at the 28-day mark, the mean reductions in serum TTR protein levels from baseline were 52 % and 87 % for the low- and high-dose groups, respectively. Following treatment, three individuals experienced mild side effects (grade 1) associated with NTLA-2001. Additionally, five out of the six patients exhibited elevated d-dimer values between 4 and 24 h after injection, but these values returned to normal in all cases by day 7 [108]. A larger phase I clinical study is currently underway, with a target enrollment of 73 patients [107].

In November 2021, Intellia Therapeutics announced a second clinical study aimed at preventing angioedema attacks in patients with hereditary angioedema (HAE). The company's NTLA-2002 CRISPR/Cas9 LNP platform is centered around the KLKB1 gene (refer to Table 2 for details). During preclinical investigations, humanized KLKB1 mice and cynomolgus monkeys were employed. The administration of just one dose of NTLA-2002 led to the editing of the KLKB1 gene by approximately 70 % in mice. Furthermore, this resulted in a reduction of over 90 % in the total plasma kallikrein protein levels [96]. A single injection also resulted in a decrease of >95 % in total kallikrein protein and activity in the monkey, along with 70 % gene editing. The technique known as Selective Organ Targeting (SORT), which utilizes a fifth permanently cationic lipid to precisely deliver CRISPR components directly to the lung, spleen, and liver of mice, has demonstrated effective tissue targeting in vivo (refer to Table 1 for details). The challenges addressed by Zhang et al. included plasmid volume for encapsulation, cell membrane penetration, cytotoxicity, and nonspecific interactions with serum or extracellular proteins, all essential for successful pDNA transportation. To reduce plasmid volume and create a compact LNP core, chondroitin sulfate and protamine were employed, thus enhancing encapsulation efficiency (as outlined in Table 2). Their LNP formulation consisted of cholesterol in conjunction with DOTAP, a permanently cationic lipid (DOPE). These two lipids improved transfection by forming electrostatic contacts with cell membranes. Additionally, DSPE-PEG modification increased stability and solubility while decreasing toxicity, extending half-life, and reducing immunogenicity [11].

6. Future direction of CRISPR/Cas9 LNP platforms

LNPs have emerged as efficient carriers for delivering CRISPR/Cas9 components, such as Cas9 mRNA, Cas9 protein, CRISPR/Cas9 RNPs, and base editors, for targeted gene editing in cells. LNPs offer advantages like biodegradability, biocompatibility, and protection of genome-editing systems. They are also easily modifiable to enhance delivery efficiency and achieve cell- or tissue-specific targeting [93]. LNP formulations for CRISPR/Cas9 gene editing must be tailored to the specific experimental application. *In vitro* applications need lipids for encapsulation and cellular uptake, while in vivo applications require lipids that enhance circulation time, escape the immune system, minimize toxicity, and interact with target cell receptors. PEG lipids can help reach hard-to-access tissues by increasing circulation time and preventing phagocytosis. Formulations for in vivo intravenous delivery should contain optimal lipid ratios. Particle size is crucial for achieving optimum CRISPR/Cas9 delivery and genome editing efficiency, as well as for colloidal formulation stability. The versatility of LNPs as a delivery platform for gene therapy is emphasized by the various factors that can be manipulated to achieve target specificity in vivo. The format of LNP-encapsulated cargo influences the approach to CRISPR/Cas9 delivery, with different lipid formulations developed to enhance delivery and increase gene-editing efficiency based on the format of the CRISPR components [90].

The rapid advancement of gene-editing technology and biomaterial science will open the door for the use of gene editing in treating clinical diseases in the near future. Overall, the use of LNPs for intracellular delivery of mRNA for cell-selective CRISPR/Cas9 genome editing holds great promise for the development of targeted therapies for genetic diseases and other applications requiring precise manipulation of the genome. Further research and development in this area are likely to lead to the advancement of novel treatments with improved safety and efficacy profiles. To fully apply this technique in a clinical setting, further in-depth preclinical studies on long-term tolerability, off-target effects, and effectiveness in large animals will be necessary.

7. Conclusion

Within the field of medicine, CRISPR-Cas9 technology has left an indelible mark, significantly bolstered by nanotechnology's role in drug delivery and treatment. This precise and potent gene-editing method has found extensive applications, ranging from disease modeling to therapeutic interventions. Nevertheless, the paramount challenge in harnessing the full potential of CRISPR-Cas9 remains the safe and effective delivery for clinical purposes. Recent strides have witnessed the emergence of various nanotechnology-based vectors, substantially augmenting cargo delivery capabilities. Nanotechnology, alongside delivery methodologies inspired by it, has not only elevated therapeutic efficacy but also substantially mitigated adverse side effects. An especially promising avenue in nanomedicine research lies in the realm of targeted CRISPR-Cas9 delivery, accomplished through the surface modification of nanoparticles—a development poised to captivate researchers. Drug nanoformulations, notably LNPs, distinguish themselves through their unique properties, derived from their diminutive size and expansive surface area. These distinctions encompass magnetic, electrical, biochemical, and optical attributes, all artfully exploited for therapeutic gain. Personalized LNPs, in particular, excel at efficiently encapsulating and safeguarding various CRISPR-Cas9 components through self-assembly. Consequently, these intelligent LNP-based delivery systems have significantly enhanced the therapeutic potential of CRISPR-Cas9 treatments, concurrently minimizing undesired off-target effects. We anticipate that future advancements in nanotechnology-based vectors will not only facilitate scalability in manufacturing but also further widen the horizons of CRISPR-Cas9-based therapeutic genome editing.

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Declaration of competing interest

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