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Synthetic multi-layer nanoparticles for CRISPR-Cas9 genome editing
Hao Tang\textsuperscript{a}, Xiaohui Zhao\textsuperscript{a}, Xingyu Jiang\textsuperscript{a,*}

\textsuperscript{a} Department of Biomedical Engineering, Southern University of Science and Technology, No. 1088 Xueyuan Rd, Nanshan District, Shenzhen, Guangdong 518055, P. R. China
* Corresponding author
E-mail: jiang@sustech.edu.cn

Abstract
The clustered regularly interspaced short palindromic repeat (CRISPR) has great potential to revolutionize biomedical research and disease therapy. The specific and efficient genome editing strongly depends on high efficiency of delivery of the CRISPR payloads. However, optimization of CRISPR delivery vehicles still remains a major obstacle. Recently, various non-viral vectors have been utilized to deliver CRISPR tools. Many of these vectors have multi-layer structures assembled. In this review, we will introduce the development of CRISPR-Cas9 systems and their general therapeutic applications by summarizing current CRISPR-Cas9 based clinical trials. We will highlight the multi-layer nanoparticles (NPs) that have been developed to deliver CRISPR cargos \textit{in vitro} and \textit{in vivo} for various purposes, as well the potential building blocks of multi-layer NPs. We will also discuss the challenges in making the CRISPR tools into viable pharmaceutical products and provide potential solutions on efficiency and biosafety issues.

Key words: genome editing, CRISPR-Cas9, non-viral vectors, multi-layer NPs, clinical transformation

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1. Introduction

In recent years, a series of programmable DNA nuclease-dependent genome editing techniques have been developed to enable efficient genetic manipulation in eukaryotes, particularly in mammalian cells [1-3]. These genome-editing techniques used for gene knock-out or knock-in predominately include meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR-Cas system [4-7]. These technologies all employ endonucleases to induce double strand breaks (DSB) at intended sites in the genome, followed by DSB repair through different mechanisms including non-homologous end joining (NHEJ), homologous recombination (HR), homology-mediated end joining (HMEJ) and microhomology-mediated end joining (MMEJ) [8]. Among these genome editing techniques, CRISPR-Cas9 is a gene editing system derived from the bacterial adaptive immune system, which is capable of combating the invasive viral genome under the guidance of short sequence RNA. CRISPR-Cas9 system is highly versatile because of its simplicity and high efficiency, moreover, any genome sequence theoretically can be edited by this system. In principle, the whole genome can be site-specifically modified at the genetic level in cells and tissues with CRISPR-Cas9 system, which further allows researchers to study the relationship between gene mutations and biological phenotypes. CRISPR-Cas9 system has driven innovative applications from basic biology research to biotechnology and medicine [9].

Precise gene manipulation has been a decades-long goal for scientists and the CRISPR is a quite young technology that is experiencing the historical development. The CRISPR was originally discovered in the genome of Escherichia coli (E. coli) in 1987 and regarded as a series of forward repeats interspaced by many short sequences. After that, similar structures were also found in a wide range of archaea and bacteria [10, 11]. Till 2005, it was observed that many spacer sequences in CRISPR originated from foreign plasmids or viruses, while the CRISPR-associated gene was reported to encode a protein with a nuclease and helicase domain [12-15]. Thus, scientists speculated the CRISPR-Cas to be a defense system in bacteria that employ antisense RNAs as memory features of past invasions [16]. In 2007, researchers firstly proved the adaptive immunity in bacteria mediated by CRISPR-Cas through infection experiments of the bacterium Streptococcus thermophilus with lytic phages [17]. In 2008, researchers discovered that mature CRISPR RNA (crRNA) can complex with Cas protein and act as a guide to interfere with virus proliferation in E. coli [18]. Besides, the DNA targeting ability of CRISPR-Cas system was also identified in 2008 [19]. Along with the elucidation of molecular mechanism, biochemical characterization and eukaryotic editing capability, CRISPR-Cas9 system has entered into an accelerated age in the past decade [1, 20, 21].

In bacteria, the CRISPR-Cas9 system works through three main processes including adaptation, expression and interference. The DNA fragments of invasive virus or plasmids can be integrated into CRISPR array as memory sequences for the defense of their re-invasion, followed by crRNA formation through transcription and maturation. Under the guidance of crRNA and partially complementary
trans-activated crRNA (tracrRNA), Cas9 acts as a restriction endonuclease for site-specific DNA recognition and cutting [20]. For gene editing, the CRISPR-Cas9 system consists of two components: a single guide RNA (sgRNA) engineered as substitute for crRNA:tracrRNA, and an endonuclease Cas9 protein. The sgRNA is about 100 nucleotides, composed of a 20-nucleotide sequence at its 5’ end and a double-stranded structure at 3’ end. The former works to combine target DNA via Watson-Crick base pairing, while the latter works to bind Cas9 protein [1, 2, 21, 22]. The targeting specificity of CRISPR-Cas9 systems is mainly determined by sgRNA, protospacer adjacent motif (PAM) and Cas9 protein. For example, the mismatch between sgRNA and targeted DNA can induce off-target effect. To reduce the off-target effect and improve the specificity of CRISPR-Cas9 system, a series of strategies have been established, for example: (1) reduce and limit the retention time of Cas9 protein inside cells [23, 24]; (2) decrease the feeding concentration of Cas9 protein [25]; (3) activate Cas9 specifically by exogenous stimulus [26, 27]; (4) neutralize electrostatic interaction between Cas9 protein and DNA [28, 29]; (5) use sgRNAs with less nucleotides (17-18 bases) can reduce off-target efficiency [30]. On the other hand, the specific and efficient gene editing also depend on the precise and effective delivery of editing payloads into intended sites, and utilize optimal carriers, for example multi-layer NPs, can maximally ensure the cargo stability to achieve the site-specific editing capability of CRISPR-Cas9 system.

Indeed, there are still many obstacles to overcome on the way to translate CRISPR-Cas9 technology into clinical applications, the safe and efficient delivery of CRISPR-Cas9 tools to target sites appears to be the most substantial one [31-33]. To date, the delivery of CRISPR-Cas9 system mainly relies on physical methods and viral vectors [34]. Physical methods mainly include electroporation, microinjection, hydrodynamic injection, ultrasonication and magnetofection, while the viral vectors mainly includes adenovirus (AV), adeno-associated virus (AAV) and lentivirus (LV) [35-37]. Generally, physical methods are more amenable for laboratory use than for clinical translation, because they are more feasible to operate at cellular level, rather than that in organisms in vivo [38]. Viral vectors also have inherent disadvantages particularly in the risk of carcinogenesis and immune responses, which severely limit the clinical translation [39, 40]. Thus, though high efficiency of CRISPR-Cas9 delivery of both physical methods and viral vectors has been established, it is still urgent to develop safe and efficient vectors to deliver genome-editing biomacromolecules. Non-viral vectors possess several advantages in delivering gene cargos, such as low immunogenicity, low restriction of cargo size and amenable for mass-production [41, 42]. Non-viral vectors, particularly multi-layer NPs, have offered promising potentials in delivering CRISPR-Cas9 system for genome editing-based applications. Multi-layer NPs can ensure the stability of gene editing cargos by packaging them inside the inner layer, while the outer layer can prevent them from degradation. Multi-layer NPs generally are more feasible to achieve the multiple functions of the non-viral vectors. Researchers try to achieve effective encapsulation and efficient transport of genome editing cargos through the rational design of the multi-layer structures. As an example, we screened more than 56 kinds
of cationic materials for Cas9/sgRNA plasmid delivery, and protamine was finally selected to condense plasmid for core formation because of its highest editing efficiency. Followed by encapsulation into a cationic lipid shell, the Cas9/sgRNA plasmid-loaded core-shell lipid system induced up to 47% successful transfection of plasmid in A375 cells [43]. In short, the rational design of these multi-layer structures will significantly influence the fate of gene editing tools, thereby the gene editing efficiency.

The Cas9 protein can be incorporated in three modes, that is DNA, messenger RNA (mRNA) and protein, correspondingly the CRISPR-Cas9 system has three general modes, that is: (1) a plasmid DNA (pDNA) that encodes both the Cas9 enzyme and the sgRNA; (2) Cas9 mRNA and sgRNA; (3) the recombinant Cas9 protein and sgRNA (RNP). Each of the modes has distinct advantages and disadvantages for genome editing. CRISPR-Cas9 pDNA is relatively low cost and has excellent stability, but it also has the disadvantages including: (1) increasing the risk of integrating the Cas9 into the genome; (2) relatively low expression efficiency, and (3) delayed onset [44, 45]. The Cas9 mRNA and sgRNA enable transient expression of Cas9, but the low stability of mRNA somewhat hinders its deployment [46, 47]. The RNP is a quite straightforward mode which has the advantages of low off-target editing, swift onset and transient duration, but the high cost of pure Cas9 protein also needed to be addressed [48, 49]. In general, the pDNA that encodes Cas9 protein and sgRNA, Cas9 mRNA and sgRNA are nucleic acid modes, thus their non-viral vectors should share similarities with those commonly used for DNA or RNA delivery. However, the CRISPR pDNA and Cas9 mRNA are quite large in size, which requires the carriers to have superior loading and encapsulation abilities. Cas9 protein has a molecular weight of about 160 kDa, which also poses challenges for the delivery of protein-based genome editing tools. Multi-layer NPs hold potentials for the delivery of CRISPR-Cas9 systems because the multi-layer structures not only provide positively charged cores to electrostatically complex and condense the cargos, but also utilize outer layers to stabilize the inner components. We have successfully employed core-shell multi-layer NPs to deliver different modes of CRISPR-Cas9 systems for different therapeutic purposes both in vitro and in vivo [43, 50, 51]. In short, multi-layer NPs provide unparalleled platforms for the delivery of genome-editing biomacromolecules.

Taking advantage of the powerful site-specific editing capability of CRISPR-Cas9 system, it has been widely applied in many different fields including disease modeling and therapy [34]. In the field of disease modeling, gene editing provides strong technical supports to understand the development of human diseases, screen drugs and seek treatments. Till now, not only mice, but also many other animals have been reported to be engineered by CRISPR technology, for example zebrafish, rabbits and monkeys [36, 52, 53]. In particular, primates are more similar to humans genetically and physiologically than other animal models, thus possess great implications for the research of human diseases. CRISPR-Cas9 technology also provides new opportunities in gene therapy through correction of genetic disorders, and some of these applications have entered into clinical trials (see in Table 1, data from www.
Chimeric antigen receptor (CAR) T cells use CAR modified T cells to specifically recognize tumor-associated antigens and activate the suppressed immuno-microenvironment for tumor therapy. CAR-T cells are the first gene transfer therapy that gained approval by the United States Food and Drug Administration (FDA) [54]. The combination of these two revolutionary technologies, CRISPR-Cas9 and CAR-T cells, possesses great potentials for the enhancement of further therapeutic efficiency and safety in immunotherapy. For example, one of the world’s first human trials of CRISPR technology has been launched in West China Hospital (Sichuan University, China) for the therapy of chemotherapy and radiation therapy resistant non-small cell lung cancer (NSCLC) via engineering patient’s T cells at genetic level through CRISPR technology (Identifier: NCT02793856). The T cells are isolated from the NSCLC patients, and engineered to knock out programmed death-1 (PD-1) gene \textit{in vitro} by CRISPR. These engineered T cells are amplified and re-inject back into the patient to achieve anti-tumor effect [55]. The results of the first phase I clinical trial has demonstrated the therapeutic safety as most adverse events (AE) were at grade 1 like fever, hyperhidrosis, and so forth, while no higher grade (3-5) AEs were observed. Based on these results, it has been possible to conduct larger scale studies to investigate effective dose and related immune response [56]. This autumn, a CRISPR-Cas9 based gene therapy developed by Editas Medicine and Allergan, EDIT-101, also will enter clinical trials for the treatment of Leber congenital amaurosis 10 (LCA10) by targeting CEP290 gene in human photoreceptor cells [57]. LCA10 is a severe retinal dystrophy caused by bi-allelic loss-of-function mutations in the CEP290 gene. The EDIT-101 is administered in participants with LCA10 \textit{via} subretinal injection, which is expected to directly edit genes in human body (distinguished from aforementioned T cell editing \textit{ex vivo}). However, viral vectors are the only choices to deliver the CRISPR-Cas9 tools for clinical purposes in currently established trials. The high transport efficiency of viral vectors should be seriously considered, but the safety concerns should never be ignored. Generally, the viral vectors have core-shell multi-layer structures, in which the outer capsid shell protects the inner nucleic acid cargos. Multi-layer NPs share structural similarities with viral vectors. Multi-functionalities of these NPs can be achieved by rationally designing the multi-layer structures. More cargos can be loaded into multi-layer NPs through the core-shell encapsulation. The bio-interactions between multi-layer NPs and target cells can be well tuned by varying the physicochemical properties of the NPs. Moreover, by fully utilizing the properties of these multi-layer NPs, even remotely controllable editing system can be developed. In short, we think that it is feasible to learn from the viral vectors to improve the efficiency of delivery and editing, and multi-layer NPs can be regarded as a series of biomimetic carriers of viral vectors. Using these NPs to deliver the CRISPR cargos can not only overcome the safety concerns caused by viral vectors, but also improve the variabilities of non-viral vectors for the delivery of the CRISPR-Cas9 system.

What are similar characteristics between the current multi-layer NPs? Is it possible to further convert the potential non-viral vectors as building blocks into multi-layer ones to improve their delivery capabilities? How to effectively accelerate these
multi-layer NPs into clinical applications? To answer these questions, we will systematically provide an overview about the non-viral vectors for CRISPR-Cas9 delivery, including multi-layer and their potential building blocks. Since the biomimetic nature of multi-layer NPs, we will emphasize their potentials in future clinical translation in terms of efficiency and safety.
<table>
<thead>
<tr>
<th>Intervention/Treatment</th>
<th>Edited Cells</th>
<th>Target Gene</th>
<th>Disease</th>
<th>Phase</th>
<th>ClinicalTrials.gov Identifier</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic: Edited T Cells Drug: CTX</td>
<td>T cells</td>
<td>PD-1</td>
<td>Metastatic NSCLC</td>
<td>Phase 1</td>
<td>NCT02793856</td>
<td>2016</td>
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<td>Edited CD34+ HSPCs</td>
<td>CD34+ HSPCs</td>
<td>CCR5</td>
<td>HIV-1-infection</td>
<td>-</td>
<td>NCT03164135</td>
<td>2017</td>
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<td>TALEN-HPV E6/E7 CRISPR-Cas9-HPV E6/E7</td>
<td>HPV16 and HPV18</td>
<td>E6/E7</td>
<td>HPV-Related Malignant Neoplasm</td>
<td>Phase 1</td>
<td>NCT03057912</td>
<td>2017</td>
</tr>
<tr>
<td>UCART019</td>
<td>CAR-T cells</td>
<td>TCR and B2M</td>
<td>B Cell Leukemia B Cell Lymphoma</td>
<td>Phase 1/2</td>
<td>NCT03166878</td>
<td>2017</td>
</tr>
<tr>
<td>Genetic: Edited EBV-CTL cells Drug: FA/CTX/IL-2</td>
<td>EBV-CTL cells</td>
<td>PD-1</td>
<td>Advanced Stage EBV Associated Malignancies</td>
<td>Phase 1/2</td>
<td>NCT03044743</td>
<td>2017</td>
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<tr>
<td>Genetic: NY-ESO-1 redirected autologous T cells Drug: CTX/FA</td>
<td>T cells</td>
<td>TCR and PD-1</td>
<td>Multiple Myeloma Melanoma Synovial Sarcoma Myxoid/Round Cell Liposarcoma</td>
<td>Phase 1</td>
<td>NCT03399448</td>
<td>2018</td>
</tr>
<tr>
<td>anti- MSLN CAR-T cells</td>
<td>CAR-T cells</td>
<td>PD-1 and TCR</td>
<td>MSLN Positive Multiple Solid Tumors</td>
<td>Phase 1</td>
<td>NCT03545815</td>
<td>2018</td>
</tr>
<tr>
<td>Genetic: MSLN-directed CAR-T cells</td>
<td>CAR-T cells</td>
<td>PD-1</td>
<td>MSLN Positive Multiple Solid Tumors</td>
<td>Phase 1</td>
<td>NCT03747965</td>
<td>2018</td>
</tr>
</tbody>
</table>
Drug: PTX/CTX

<table>
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<th>Type</th>
<th>Indicator</th>
<th>Target</th>
<th>Disease</th>
<th>Stage</th>
<th>ID</th>
<th>Year</th>
</tr>
</thead>
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<td>CTX001</td>
<td>CD34+ hHSPCs</td>
<td>BCL11A</td>
<td>β-Thalassemia</td>
<td>Phase 1/2</td>
<td>NCT03655678</td>
<td>2018</td>
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<tr>
<td>Edited Patient-specific iHSCs</td>
<td>iHSCs</td>
<td>HBB</td>
<td>Thalassemia</td>
<td>Early Phase 1</td>
<td>NCT03728322</td>
<td>2018</td>
<td></td>
</tr>
<tr>
<td>Universal Dual Specificity CD19 and CD20 or CD22 CAR-T Cells</td>
<td>CAR-T Cells</td>
<td>CD19 and CD20 or CD22</td>
<td>B Cell Leukemia</td>
<td>Phase 1/2</td>
<td>NCT03398967</td>
<td>2018</td>
<td></td>
</tr>
<tr>
<td>Genetic: XYF19 CAR-T cells</td>
<td>CD19+ CAR-T cells</td>
<td>HPK1</td>
<td>Relapsed or refractory CD19+ leukemia or lymphoma</td>
<td>Phase 1</td>
<td>NCT04037566</td>
<td>2019</td>
<td></td>
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<tr>
<td>Drug: CTX/FA</td>
<td>T cells</td>
<td>CD.9</td>
<td>Non-Hodgkin Lymphoma</td>
<td>Phase 1/2</td>
<td>NCT04035434</td>
<td>2019</td>
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<td>CTX110</td>
<td>Photoreceptor cells</td>
<td>CEP290</td>
<td>LCA10</td>
<td>Phase 1/2</td>
<td>NCT03872479</td>
<td>2019</td>
<td></td>
</tr>
</tbody>
</table>

2. Multi-layer NPs for CRISPR-Cas9 delivery
2.1 Gold nanoclusters (AuNC)-assisted lipid NPs

Gold-based nanomaterials, for example AuNC and gold nanoparticles (AuNP), possess excellent physicochemical stability and good biocompatibility, thus gold nanomaterials are regarded as promising non-viral materials for the delivery of genes and proteins. Lipid NPs are three-dimensional spherical vesicles formed by assembly with the help of lipid molecules. Taking advantages of delivery capabilities of gold-based nanomaterials and lipid NPs, we have prepared AuNC and AuNP assisted multi-layer lipid NPs to deliver different CRISPR-Cas9 modes.

AuNC is a class of gold based nanomaterials that exists as gold atom aggregates with the particle diameter smaller than 3 nm. AuNC has large specific surface area. More importantly, AuNC has better renal clearance abilities than AuNP, since the kidney threshold is about 5 nm [58, 59]. High biocompatibility and biosafety have made AuNC an ideal non-viral delivery material. We have previously reported the assistance of oligoarginine-capped AuNC for the delivery of small interfering RNA (siRNA) of nerve growth factor (siRNA NGF) both in vitro and in vivo, targeting the nervous microenvironment of pancreatic cancer [60]. The complex of AuNC and siRNA NGF exhibited increased serum stability, elongated blood circulation time, enhanced cellular internalization and tumor accumulation, effective therapeutic effect in multiple pancreatic tumor models, demonstrating the profound vector advantages of AuNC. Inspired by this, we also utilized AuNC to deliver Cas9 protein and sgRNA plasmid to treat melanoma by targeting Polo-like kinase-1 (Plk1) (Figure 1) [51]. To improve the nucleus targeting capability of AuNC, we used the HIV-1-transactivator of transcription peptide (TAT peptide) to cap AuNC (TAT-GNCs), followed by electrostatic complexation with Cas9 protein and sgRNA plasmid [61]. We capsulated the negatively charged ternary complex core into an anionic lipid shell which was formulated by 1,2-dioleyl-3-trimethylammoniumpropane (DOTAP), dioleoyl-phosphatidylethanolamine (DOPE) and cholesterol at the molar ratio of 0.8:1:0.5, further we also used 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(carboxy(polyethylene glycol)) (DSPE-PEG) to post-modify the lipid vector (termed as LGCP) (Figure 1A). The LGCP appeared 104 nm in diameter with about 35 mV surface charge. The LGCP was quite stable and one single LGCP can load about 1320 AuNCs. As evidenced, large percentage of Cas9 proteins (25 %) and sgPlk1 (22 %) can be simultaneously delivered into the nucleus for gene editing under the guidance of TAT peptide. Moreover, the endosomal/lysosomal release of cargo in LGCP can be achieved because DOTAP can induce the disassembly of LGCP and destabilization of endosome membrane [62, 63]. The in vitro gene editing efficiency can reach 26 % in Plk1 locus, which further effectively inhibited tumor cell growth (Figure 1B). Besides, no significant off-target mutations were observed in 10 potential sites. Moreover, in situ injected LGCP can specifically down-regulate the Plk1 in about 24 % tumor cells, which further inhibited ≈ 75 % melanoma tumor progression (Figure 1C). More recently, we further modified the LGCP to realize triple-targeting delivery of the RNP in vivo for PCSK9 knockout (Figure 2) [50]. PCSK9 is a key metabolic regulator of
low-density lipoprotein cholesterol (LDL-C), which is closely related to the coronary heart disease (CHD) [64-66]. PCSK9 can be a promising therapeutic target for LDL-C induced CHD. We employed the nuclear localization signal (NLS)-tagged Cas9 protein and sgPCSK9 to mix with TAT-GNCs to form the anionic complex (TAT-GNCs/Cas9/sgPCSK9). Similarly, we loaded the TAT-GNCs/Cas9/sgPCSK9 into lipids formulated by DOTAP and DOPE. Because PCSK9 is expressed in and secreted from liver, we post-modified the GNCs loaded lipids with 4-aminophenyl β-D-galactopyranoside (Gal)-modified polyethylene glycol phospholipid (Gal-PEG-DSPE) instead of DSPE-PEG, aiming to target asialoglycoprotein receptor (ASGPR) on hepatocytes (Figure 2A) [67]. The diameter of the resulting hybrid lipid NP (termed as Gal-LGCP) was 106 nm and the zeta potential was 34 mV, both of which remained stable in multiple medium solutions. Owing to the triple-targeting capability (cell targeting, cell membrane penetration, and subcellular targeting) of Gal-LGCP, the RNP can be effectively delivered into nucleus through several transporting barriers. In vitro, the Gal-LGCP induced about 60% PCSK9 knockout in Hepa 1-6 cells while no off-target mutagenesis was observed in 10 potential sites. In vivo, the Gal-LGCP can efficiently target to liver cells. Moreover, Gal-LGCP specifically knocked down the PCSK9 in liver cells further downregulating the LDL-C level of approximately 30 % post the injection 49 days (Figure 2B). Collectively, the Gal-LGCP we proposed have profound potentials in preventing and treating cardiovascular diseases safely and effectively.
Figure 1. AuNC assisted multi-layer NPs for CRISPR-Cas9 based tumor therapy. (A) Schematic diagram of the synthesis process of the LGCP. (B) The editing of Plk1 locus in vitro induced by LGCP on A375 cells examined by T7E1 assay and western blot assay. (C) Therapeutic editing effects of A375 tumor model on mice by LGCP and western blot analysis of the tumor tissues. Reprinted with permission from Ref. [51]. Copyright (2017) The Authors. Published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

2. AuNP assisted lipid NPs

AuNP is also often used as an excellent non-viral gene vector because of its unique physicochemical properties [68]. An arginine-modified AuNP was used to directly deliver RNP into cytoplasm by membrane fusion, which allowed the gene editing efficiency of this formulation to be as high as 29% (in AAVS1 locus) and 30% (in PTEN locus) [69, 70]. Researchers developed thiolated DNA-coated AuNPs
(CRISPR-Gold) to encapsulate CRISPR-Cas gene editing tools and deliver them into directly in cells (Figure 3A) [71, 72]. The CRISPR-Gold was generated through encapsulating the complexes of Cas9 (or Cpf1) and AuNPs by endosomal disruptive polymers. The AuNP was surface modified with DNA oligonucleotides and appeared 15 nm in diameter. The CRISPR-Gold has been successfully utilized to treat Duchenne muscular dystrophy (DMD) and fragile X syndrome (FXS) in mice models. This treatment did not cause detectable levels of toxicity, even after repeated injections. Recently, colloidal AuNPs (19 nm) were reported to *ex vivo* deliver the entire CRISPR payload (AuNP/CRISPR) into primary human blood progenitors and efficiently function the gene editing in multiple loci of therapeutic interests without toxicity, which opened potential treatment modalities for numerous diseases [73].

One excellent property of AuNP is the photo-thermal transfer capability induced by localized surface plasmon resonance, which is widely utilized in photo thermal therapy or as a stimulus for cargo release [74, 75]. Based on this, we reported a coordinated strategy that combined AuNP and lipid formulations to deliver Cas9-sgPlk1 plasmid (CP) for tumor (melanoma) therapy (Figure 3B) [76]. We synthesized the AuNP (about 20 nm) using glutathione and TAT as the reductant and cationic motif respectively. The CP was complexed with the TAT-modified AuNP. The cationic lipid shell for complex encapsulation was composed of DOTAP, DOPE, cholesterol, DSPE-PEG. The overall AuNP-based lipid formulation (termed as LACP) was 101 nm in diameter with good dispersity while the zeta potential was about 36 mV. Unlike the LGCP we discussed above, only about 4-15 AuNPs were loaded inside the lipid shell. The plasmid encapsulation efficiency of LACP was as high as 97 %, moreover, the plasmid release efficacy can reach 80 % under the laser (514 nm) irradiation. *In vitro*, laser irradiation assisted the cytosol release of CP after the endocytosis, after which, the CP can be successfully delivered into the cell nuclei. In this way, about 60 % Plk1 in A375 cells were down-regulated, which indicated the successful delivery and gene editing. When treated the xenograft model of human melanoma through *in situ* injecting LACP, the tumor inhibition rate achieved 58 % under the laser irradiation, which demonstrated to be the most effective treatment compared with other groups. Overall, the AuNP core was regarded not only as core vectors but also triggered for cargo release, which paved a new horizon for AuNP-based CRISPR/Cas9 delivery systems. Similarly, researchers also used gold nanorod-based multilayer NPs (termed nanoCRISPR) to achieve optogenetically activatable and spatially controllable genome editing of CRISPR-Cas9 [77]. The nanoCRISPR composed of cationic polymer-decorated gold nanorod and Cas9 plasmid which has a heat-inducible promoter. The nanoCRISPR was assembled layer by layer. The gold nanorod not only served as the inner core but also acted as photothermal converter of external light. By taking advantage of the photothermal conversion capability of gold nanorod upon the light irradiation in second near-infrared (NIR-II) optical window, the nanoCRISPR can locally trigger the transcription of Cas9 plasmid, thereby activate the genome manipulation. Programmable genome editing can be finely controlled by tuning optogenetic conditions, for example exposure time and irradiation time. The deep penetration feature of NIR-II light enabled nanoCRISPR to
activate at deeper tissue for therapeutic editing while minimizing the off-target effects. Collectively, these multilayer NPs enabled optogenetic control of genome editing of CRISPR-Cas9 with high spatial accuracy, which will further expand the applications of CRISPR-Cas9.

**Figure 3.** AuNP assisted multi-layer NPs for the delivery of CRISPR-Cas9 tools. (A) Schematic diagram CRISPR-Gold synthesis. Reprinted with permission from Ref. [72]. Copyright (2018) Springer Nature. (B) Synthesis process of LACP for the delivery of Cas9-Plk1 pDNA and schematic diagram of laser-enhanced knock-out of Plk1 by LACP in A375 cells. Reprinted with permission from Ref. [76]. Copyright
Lipid NPs have widely been used in drug delivery, and expected to be the preferable carriers for clinical translation. Several lipid NPs-based pharmaceutical formulations encapsulating therapeutic agents within the lipid membrane, such as Doxil® and Myocet®, have been already on the market. Some lipid NPs-based nucleic acid delivery systems aiming to treat cancers have also entered clinical trials [78, 79]. In the case of delivering CRISPR-Cas9 system, however, only few kinds of commercial transfection kits have been established for in vitro assay so far, mainly including Lipofectamine CRISPRMAX and transfection lipid products that developed for plasmid or siRNA delivery, like Lipofectamine™, TurboFect™ and Stemfect™. As one of the most commonly used non-viral carriers for bio-therapeutics, it is quite promising to develop multi-layer lipids NPs to deliver CRISPR-Cas9 systems for clinical purposes. Lipid NPs-mediated cargo delivery requires the NPs to go through a series of extracellular and intracellular barriers. Physicochemical parameters of lipid NPs (for example rigidity, lipid composition, size) can affect the interaction between NPs and target cells. Our previous works have demonstrated the key role of rigidity of lipid NPs [80, 81]. We realized that the cellular uptake efficiency of lipid NPs can be dramatically regulated by rigidity. Lipid NPs with a rigid core can transport through the cell membrane faster and easier than those that are soft via a total energy minimization mechanism [80]. Similarly, lipid NPs with flexible lipid shell can enter cells less efficiently than the ones with rigid shell [81]. Such results have driven us to mechanically design and develop favorable nano-systems for therapeutic applications, for example overcoming drug resistance [82]. Moreover, molecular structure of lipids can also influence the efficiency of delivery of lipid NPs, multiple parameters including pKa and stability can serve as a collective to predict their efficiency of delivery both in vitro and in vivo [83-85]. A series of prescreening works have identified the criteria in structure and pKa for efficient RNAi therapeutics delivery. For example, lipid molecules with a tertiary amine tail groups at one end or lipid NPs with pKa lower than or equal to 5.4 appear to induce higher possibility in efficient gene silencing in vitro and in vivo [85]. By taking advantage of the integration capability and functional tunability of lipid NPs, we have constructed AuNC and AuNP based multi-layer NPs to achieve the efficient delivery of CRISPR cargos. We will further rationally regulate the physicochemical parameters of these multi-layer NPs to tune their fates both in vitro and in vivo, aiming to deliver CRISPR-Cas9 systems for different therapeutic purposes.

2.3 Up-conversion NPs (UCNPs)-assisted NPs

Lanthanide-doped upconversion nanoparticles (UCNPs) represent a kind of promising nano-transducers that has the capability to converse near-infrared (NIR) radiation into UV or visible light, which provides a tool to remotely manipulate biological processes [86]. Recently, researchers achieved effective CRISPR RNP loading and NIR light controlled gene editing by UCNPs assisted multi-layer NPs. The UCNPs were covalently conjugated with CRISPR RNP via a UV-cleavable linker.
4-(hydroxymethyl)-3-nitrobenzoic acid (ONA), by which to realize remote manipulation of gene editing for tumor therapy by targeting Plk1 gene (Figure 4) [87]. Briefly, the synthesized oleate-capped UCNPs were coated with silica shell to enhance water solubility and biocompatibility, the UCNPs were then treated with carboxylation reagents for ONA coupling via esterification, and Cas9 proteins were conjugated on the surface via carbodiimide chemistry followed by sgRNA complexation. At last, polyethylenimine (PEI) was applied for overall coating to enhance endosomal escape. As designed, the resulting UCNPs (termed as UCNPs-Cas9@PEI) can realize the NIR light trigged release of CRISPR Cas9 editing tools. In vitro, UCNPs-Cas9@PEI plus NIR light (980 nm) irradiation resulted in the Plk1 knockout efficacy to be 32 %, which induced remarkable inhibition of cell proliferation and apoptosis. In vivo, UCNPs-Cas9@PEI plus NIR light (980 nm) irradiation group exhibited 74 % tumor inhibition rate, demonstrating the high potency of the NIR triggered gene editing systems. Nevertheless, the therapeutic effect can only be achieved through intratumoral administration, not by intravenous injection. Thus, similar UCNPs that systemically target tumoral or metastatic nodules are still under development.

Figure 4. Schematic diagram of the UCNP-based CRISPR-Cas9 delivery system for NIR light triggered gene editing. Reprinted with permission from Ref. [87]. Copyright (2019) The Authors, some rights reserved. Exclusive licensee American Association for the Advancement of Science. Distributed under a Creative Commons Attribution NonCommercial License 4.0.

2.4 Nanocapsule

Polymers are widely applied in the field of drug delivery, and several polymers are currently in the clinical trials [88]. Accordingly, favorable polymeric formulations with preferable pharmacokinetics and efficiency of drug delivery for diseases therapy
can be obtained by rational designing the properties of polymer NPs, including architectures and compositions [89, 90]. Moreover, the synthetic polymers possess the advantages of monomer-derived variabilities, which means that the tailored properties can be integrated in a single delivery vector by using diverse functional monomers [91]. Taking advantage of this, researchers recently reported a 25 nm polymeric nanocapsule that possess multi-layer structures to coat RNP complex for in vitro and in vivo somatic gene editing (Figure 5) [92]. The nanocapsule can be synthesized and further modified through in situ free-radical polymerization between vinyl monomers, for instance, the cationic moieties for RNP complexation, biodegradable disulfide bonds for glutathione (GSH) induced cargo release, PEG methyl ether (mPEG) for colloidal stability and specific ligands for targeting delivery. The synthetic multi-layer nanocapsule realized the delivery of RNP in a controlled stoichiometry manner by loading one RNP inside one nanocapsule, which limits the safety concerns resulting from variabilities in carried cargo. Further, efficient targeted gene editing was achieved in vivo in murine retinal pigment epithelium tissue and skeletal muscle after in situ administration, without causing apparent cytotoxicity. This type of multi-layer nanocapsules has been demonstrated to be a promising vector for safe and efficient delivery of the CRISPR-Cas9 system.

Figure 5. A biodegradable nanocapsule for the delivery of the Cas9 RNP complex. (A) Schematic illustration for the preparation of the covalently cross-linked and intracellularly biodegradable nanocapsule. (B) Intracellular fate of the nanocapsule and GSH triggered RNP release. Reprinted with permission from Ref. [92]. Copyright (2019) Springer Nature.
2.5 Nanolipogel

Lipogels possess potential in the delivery of biomacromolecules because they can localize the cargos stably inside the gel core. Recently, a multi-layer deformable and tumor targeted nanolipogel (tNLG) was reported to co-deliver three CRISPR-Cas9 plasmids for the therapeutic genome editing of triple-negative breast cancer (TNBC) (Figure 6) [93]. This is also an example that utilized non-cationic carrier to deliver CRISPR-Cas9 system. The tNLG featured a deformable core-shell structure with a diameter of 111 nm, in which, the innermost layer of tNLG was the biodegradable alginate hydrogel that encapsulated the plasmids. The plasmids-loaded hydrogel was then coated with non-cationic lipid bilayer constructed by zwitterionic 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and anionic DSPE-PEG. ICAM-1 antibody was further used to engineer the outermost layer to mediate the selective interaction between tNLG and TNBC cells. The ICAM-1 antibody guided the tNLG to enter the TNBC cells via receptor-mediated membrane fusion pathway directly into cytosol without endosome entrapment. As a result, the systemically administration of tNLG efficiently knocked out > 81% Lcn2 in TNBC and inhibited 77% of the tumor growth in an orthotopic TNBC model.

Figure 6. Schematic illustration of tNLG structure for the delivery of CRISPR-Cas9 pDNA and bio-mechanisms of in vivo CRISPR based therapeutic genome editing. Reprinted with permission from Ref. [93]. Copyright (2019) Published under the PNAS license.

2.6 Polymeric NPs

It is feasible to condense CRISPR cargos into solid nano-complexes by the electrostatic interactions between cationic polymers and cargos themselves, and
Additionally encapsulate the complexes by amphiphilic polymers to form multi-layer NPs that can enhance the *in vivo* delivery capacities of these non-viral vectors. Cationic polypeptides which has α-helical conformation can serve both as effective gene vectors and cell penetrating agents [94-96]. Recently, the α-helical polypeptide poly(γ-4-((2-((piperidin-1-yl) ethyl) aminomethyl) benzyl-L-glutamate) (PPABLGL) was evidenced to be a robust carrier for Cas9 pDNA and sgRNA (Figure 7A) [97]. The NP formulation (P-HNPs) was prepared as follows: PPABLGL bound and condensed Cas9 pDNA and sgRNA into nano-complexes, followed by PEGylation using PEG-Polythymine40 (PEG-T40). The cell penetrating ability of PPABLGL efficiently help P-HNPs entering different kinds of cells, escaping from endosomes and transporting cargos into nucleus. *In vitro*, the P-HNPs exhibited much higher cellular internalization efficiency (67%) than that of Lipofectamine 3000 (33%). Accordingly, the P-HNPs can more effectively cause insertion-deletion events (40%) than other commercial agents did in the case of knocking out eGFP in U2OS.EGFP cell lines. Meanwhile, the P-HNPs did not cause high off-target effect (< 2.8%). Also, P-HNPs can achieve both gene insertion and activation *in vitro* and *in vivo*. Moreover, researchers used P-HNPs to deliver Cas9 plasmid/sgDNA (abbreviated as P-HNPCas9+sgPlk1) targeting Plk1 for anticancer therapy. *In vivo*, the P-HNPPCas9+sgPlk1 achieved 35% gene disruption in Plk1 locus after intratumoral injection in HeLa xenograft tumor, thereby inhibited >71% tumor growth and prolonged the survival to 60% till 60 days. This work showed the promising capabilities of chiral materials for CRISPR-Cas9 delivery.

More recently, researchers used phenylboronic acid (PBA) to modify low-molecular-weight PEI (2081 Da) for the delivery of CRISPR-Cas9 pDNA to activate miR-524 gene (Figure 7B) [98]. The modified cationic polymer PEI-PBA condensed pDNA into a core structure, which was encapsulated into core-shell NP (MDNP) by acid-triggered charge reversal polymer 2, 3-dimethylmaleic anhydride (DMMA)-modified poly(ethylene glycol)-b-polylysine (mPEG113-b-PLys100/DMMA). The MDNP charged negatively in bloodstream, but turned to positive after DMMA dissociation in tumor microenvironment, which further exposed the PEI-PBA/pDNA core due to the electrostatic repulsion-induced shell detachment. The PBA structure eventually enhanced the internalization of PEI-PBA/pDNA core into MDA-MB-231 cells via binding with sialic acid on the cell membrane and the PEI helped to trigger the endosomal escape and release the pDNA. As a result, the MDNP/dCas9-miR-524 formulation significantly retarded the tumor growth in mice via upregulating miR-524 in tumors, demonstrating the feasibility of MDNP. Similarly, researchers also used PEG-detachable NPs to deliver Cas9 RNP with a combination of sgRNAs (nanoRNP), providing a feasible strategy to effectively overcome tumor heterogeneity [99]. By targeting signal transducer and activator of transcription 3 (STAT3) and Runt-associated transcription factor 1 (RUNX1), the nanoRNP down-regulated the expressions of STAT3 by 48% and RUNX1 by 50% in heterogeneous tumors *in vivo*, exhibiting effective inhibition of tumor growth.

The core-shell polymeric NPs also has the potential to deliver other CRISPR systems (e.g., the CRISPR-Cas13a system) for therapeutic applications. Recently,
researchers used a PEG-\textit{b}-PLys derived dual locking (responding to the pH and the \( \text{H}_2\text{O}_2 \)) NP (DLNP) to deliver CRISPR-Cas13a pDNA that targeted programmed death-ligand 1 (PD-L1) for the activation of T-cell mediated antitumor immunity and reconstruction of immunosuppressive tumor microenvironment [100]. This work potentially provided an efficient platform for CRISPR based cancer immunotherapies.

\textbf{Figure 7.} Multi-layer polymeric NPs for the delivery of CRISPR Cas9 tools. (A) Schematic formation of P-HNPs and the intracellular activity of Cas9 expression plasmid/sgRNA in genome editing. Adapted from Ref. [97]. Copyright (2018) the Author(s). Published by PNAS. Distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0. (B) Schematic illustration for the preparation of MDNP and multistage delivery processes after intravenous administration. Reprinted with permission from Ref. [98]. Copyright (2018) The Authors. Published by WILEY- VCH Verlag GmbH & Co. KGaA, Weinheim.
The targeting delivery of CRISPR-Cas9 components into specific type of cells can be achieved by employing multi-layer NPs using highly specific polymers. For CRISPR-Cas9 technology, the delivery with high selectivity and specificity is of principal importance, which will immensely broaden its applications for safe genome editing. Generally, the design of targeted delivery system mainly depends on the high binding affinity between actively target moieties and overexpressed receptors on the cell surface. Taking advantage of the highly specific affinity of aptamer seem to be a feasible strategy. Recently, nucleolin-specific aptamer (AS1411) and MUC1 specific aptamer has been utilized to decorate the delivery vectors for enhanced endocytosis and genome editing efficiency in cancer cells [101, 102]. Besides, some delivery materials themselves have the abilities of active targeting, for example, hyaluronic acid (HA) can specifically bind to CD44 receptors. Based on this, researchers employed HA to modify the protein-based drug, ribonuclease A (RNase), with the help of lipid like molecules [103]. Through this strategy, the RNase-HA demonstrated enhanced cancer cell targeting and killing abilities than that of RNase itself. Further, researchers conjugated tandem peptide R8-RGD to HA, the dual-targeting vector exhibited multi-layer structures and enabled to efficiently deliver CRISPR-Cas9 plasmid that targeting the MutT Homolog1 (MTH1) for ovarian cancer therapy both in vitro and in vivo[104]. The dual-targeting “core-shell” artificial virus exhibited higher transfection efficiency than that of commercial SuperFect, Lipofectamine 2000, and Lipofectamine 3000, with minimum side effects.

3. Novel building blocks for multi-layer NPs

This section outlines novel building blocks which might themselves deliver CRISPR-Cas9 cargos; they can potentially be constituents as multi-layer structures that can also be used for the transportation of genome editing tools. These building blocks include lipid or lipid-like NPs with aqueous cavities inside the lipid layer, and nanocomplexes formed by the interaction between cationic polymers and anionic genome editing cargos. We will exemplify these carriers in their functions in delivering the tools for genome editing and underscore their potential as building blocks of multi-layer NPs, aiming to drive their potential conversions into multi-layer ones.

3.1 Lipidoid NPs

A class of cationic lipid-like molecule (lipidoid) NPs can be utilized to deliver CRISPR-Cas9 tools. The lipidoids mainly consist of amine heads and aliphatic tail chains in structure. Generally, the amine heads function as cationic templates to complex with anionic cargos, while the aliphatic tails function to improve the stability or facilitate the cargo release. These lipidoid NPs possess a single lipid layer to protect the cargos are loaded inside the aqueous cavity. Previously, the lipidoid NPs have been employed as intracellular carriers for conventional gene cargos like DNA, siRNA, microRNA [105, 106]. The lipidoids were combinatorially synthesized through Michael addition reaction and ring-opening reaction between commercially available electron-rich amines with lipophilic acrylates and epoxides, respectively [107]. Such facile chemistry also enables the combinatorial study of the
structure-activity relationships to screen and discover the optimal carrier. In one study, researchers prepared 12 bioreducible lipidoid NPs to deliver anionic RNP into HEK293 cell lines for efficient gene editing [108]. The helper lipidoids were synthesized from acrylates and amines (including primary and secondary amines) through Michael addition reaction, and exhibited bioreducible abilities owing to the existence of disulfide bond in its tail structure which further facilitated easy endosomal escape of RNP and its following nucleus entrance. The overall bioreducible lipidoid NPs were self-assembled with the synthetic lipidoids, cholesterol, DOPE, and C16-PEG2000-ceramide at a wt/wt ratio of 16:4:1:1, the diameter of the lipidoid NPs increased from 74 to 292 nm after RNP complexation, while the zeta potential decreased from 12.5 to -9.1 mV (3-O14B as the example). In vitro studies, lipidoid NP 3-O14B, 4-O14B and 6-O14B exhibited comparable EGFP knock-down efficiency (70 %) to that of commercial Lipofectamine 2000 in HEK293 cells by delivering the RNP. Further, the lipidoids cause lower toxicity and immunogenicity than that of Lipofectamine products relevant to in vivo applications [109].

The chemical composition of amine heads and aliphatic tail chains closely related to the efficiency of delivery. To specifically elucidate the relationship between chalcogen-containing lipidoids (R-O17X; R = amine head group; X=O, S, or Se) and their efficiency of intracellular RNP delivery, researchers similarly utilized the combinatorial library strategy to screen chalcogen-containing lipidoids for RNP in vitro and in vivo delivery (Figure 8A) [110]. The lipidoid R-O17X were prepared by reacting alkyl-acrylate tails bearing chalcogen esters with various amines through Michael addition reaction, and the lipidoid NPs were fabricated via the combination of sonication and vortexing. Three formulations (100-O17Se, 80-O17Se, 77-O17Se) exhibited higher efficiency of delivery of supercharged Cre recombinase ((-30) GFP-Cre) into HeLa-DsRed cells than that of Lipofectamine 2000 (~ 31 %), with the rate of GFP positive cells at 42 %, 39 %, and 37 % respectively. The membrane disruption ability directly regulated the efficiency of protein delivery of these lipidoid NPs. Such results indicated that lipidoids with O17Se tails are more efficacious NPs in delivering proteins than that of O17S. As a result, lipidoid NPs 76-O17Se (50 %), 80-O17Se (58 %), 81-O17Se (55 %) and 400-O17Se (57 %) exhibited higher GFP knockout efficiency than others, which were comparable to that of Lipofectamine 2000 (~ 63 %) by delivering the CRISPR Cas9 RNP. Meanwhile, 76-O17Se and 81-O17Se also showed low toxicity. From this result, it seemed reasonable to push such NPs forward into in vivo applications in terms of efficiency and biocompatibility issues.

Indeed, the lipidoid NPs have been recently employed for delivery of Cas9 mRNA and sgRNA. These lipidoid NPs can achieve fast and efficient genome editing both in vitro and in vivo by simultaneously delivering Cas9 mRNA and sgRNA into cells (Figure 8B) [111]. Likewise, the lipidoid NPs were formulated with lipidoid, cholesterol, DOPE, and DSPE-PEG2000 at a wt/wt ratio of 16:8:4:1, and the disulfide bonds contributed to the GSH-triggered NPs degradation and cargo release. In this work, lipidoid NPs BAMEA-O16B and PPPDA-N16B exhibited comparable
efficiency of luciferase mRNA delivery to Lipofectamine 2000 with high biocompatibility. The leading lipidoid BAMEA-O16B was reported as a general mRNA nanocarrier, because it can effectively transport mRNA in different length into cells. After the endocytosis processes, the disulfide bond of BAMEA-O16B facilitated endosome escape and RNA release. Thus, delivering the RFP mRNA by BAMEA-O16B can upregulate expression of RFP by four times. The sgRNA is about 103 nucleotides in size, while Cas9 mRNA is around 4500 nucleotides in size. The complex between BAMEA-O16B and Cas9 mRNA/sgRNA can be formulated into well-dispersed lipidoid NPs around 230 nm through electrostatic interaction. For GFP knockout in HEK-GFP cell lines in vitro, the GFP knockout efficiency can be increased with the Cas9 mRNA feeding enhancement, and 90 % knockout efficiency can be achieved when the concentration of Cas9 mRNA reached 160 ng mL$^{-1}$. It should be noted that very fast gene editing (40 % GFP knockout within 24 h) can be observed because of the high efficiency of mRNA delivery using BAMEA-O16B. The authors also employed BAMEA-O16B to co-deliver Cas9 mRNA and sgRNA targeting HPV18 (abbreviated as sgRNA$_{HPV18}$) to inhibit the cell proliferation of HeLa cells. The cell viability assay demonstrated that BAMEA-O16B/Cas9 mRNA/sgRNA$_{HPV18}$ can effectively inhibit HeLa cell growth via HPV18 knockout. To further confirm the in vivo Cas9 mRNA delivery capability of BAMEA-O16B, a key regulatory gene in lipid metabolism (PCSK9) was chosen as the therapeutic target for in vivo gene editing to reduce the risk of cardiovascular disease [112]. The biodistribution study showed that BAMEA-O16B/Cas9 mRNA/sgRNA mainly accumulated in liver after tail intravenous injection. Moreover, the mouse serum PCSK9 level can be reduced down to 20 % of the control group, without causing signs of inflammation or hepatocellular injury, as revealed by haematoxylin and eosin (H&E) staining and serum analysis.
Figure 8. Cationic lipidoids for CRISPR-Cas9 delivery. (A) Synthetic route and chemical structures of chalcogen-containing lipidoids and their efficiency of delivery of Cas9:sgRNA. Adapted with permission from Ref. [110]. Copyright (2018) Elsevier.
A novel class of non-cationic lipidoid NPs were reported to deliver the genome-editing proteins [113]. These lipid NPs were composed of nitrilotriacetic acid (NTA)-containing lipidoids, and fabricated with the formulation of helper lipids cholesterol, DOPE, and DSPE-PEG2000. Various His-tagged proteins including CRISPR-Cas9 RNP can be delivered into mammalian cells by these lipid NPs. The Cas9 proteins were modified with nuclear localization sequence and 6xHis tags. After the complexation with RNP, NPs with diameters of 150-350 nm were formulated. The NTA-EC16-based lipid formulations performed greater efficiency of gene-editing delivery than NTA-O16B and NTA-O17O. This work demonstrated the possibilities of delivering proteins via using non-electrostatic interactions, regardless of the relative low efficiency of delivery.

3.2 Poly (ethylene glycol)-poly (lactic-co-glycolic acid) (PEG-PLGA) lipid NPs

PEG-PLGA has been widely used in the field of biomedical engineering including drug delivery [114, 115]. PEG-PLGA is an amphiphilic polymer which consists of PEG and PLGA blocks. Till now, many PLGA-based drug formulations for therapeutic aims have already been approved by FDA or entered into clinical trials [116]. Many hydrophobic drugs (e. g. doxorubicin and paclitaxel) can be efficiently loaded with PLGA and form stable NPs [117]. In addition, a series of works reported also demonstrated the capabilities of PLGA-based polymers for hydrophilic nucleic acid delivery for gene therapy [118]. PEG-PLGA along with cationic lipid molecules were used to produce cationic lipid NPs (e.g. cationic lipid-assisted nanoparticles (CLAN)) by double emulsification methods, and hydrophilic therapeutics can be trapped into the inner water cavity [119-121]. Researchers used these PLGA based lipid NPs to deliver gene editing tools into different target cells both in vitro and in vivo. The CLAN mainly can deliver either the plasmid encoding Cas9 protein and sgRNA or the Cas9 mRNA/sgRNA, it seems rather difficult for CLAN to encapsulate Cas9 protein into the aqueous cavity, probably limited by the large size of Cas9 protein. That is because the loading capacity of CLAN is mainly determined by the aqueous cavity, however, it is quite difficult to encapsulate Cas9 proteins or the RNP into the cavity efficiently during preparation of CLAN. We think introducing a multi-layer structure when preparing the CLANs may be a feasible strategy to effectively anchor the protein cargos tightly inside the cavity by providing not only positive charges but also physical supports.

The CLANs were recently employed to deliver CRISPR-Cas9 plasmid (pCas9) or Cas9 mRNA and sgRNA to neutrophils or macrophages for inflammatory diseases treatment by genome editing [122]. Inflammation is the immune responses that closely related to development of many diseases, for example type 2 diabetes (T2D) and cancer [123, 124]. To modulate the neutrophil-related inflammation (high-fat diet induced T2D) at genomic level, the pCas9 was directly delivered into neutrophils in
vivo by N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryoxycarbonyl-aminoethyl) ammonium bromide (BHEM-Chol) assisted CLAN (Figure 9A) [125]. The surface properties including surface charge and PEG density of CLAN can significantly influence their in vivo fate and efficiency of cargo delivery [126]. In vivo screening showed that the CLAN45 with lower surface PEG density (69 %) and higher surface charge (32 mV), produced with PEG5K-PLGA11K, PLGA8K, and BHEM-Chol at a wt/wt ratio of 15.6:8.3:0.3, can accumulate into neutrophils better than other formulations. CLAN45 encapsulating pCas9 that targeting neutrophil elastase (NE) (abbreviated as CLANpCas9/sgNE) was used to down-regulate NE level both in the mice liver tissue and epididymal white adipose tissue (eWAT), aiming to simultaneously improve the glucose tolerance and insulin sensitivity in high-fat diet induced T2D model mice. The biodistribution study showed that CLANpCas9/sgNE accumulated significantly higher in liver than that of other organs. Flow cytometry results indicated that in the liver, neutrophils internalized more CLANpCas9/sgNE than Kupffer's cells. Two days after the intravenous injection of CLANpCas9/sgNE, the indel frequency in neutrophils of eWAT and liver was respectively 20 % and 27 %.

Off-target effect analysis showed that the five predicted off-target effects were remarkably lower than on-target effect, which demonstrated the safety of CLANpCas9/sgNE. The treatment with CLANpCas9/sgNE decreased the NE expression, thereby reduced neutrophils infiltration, down-regulated pro-inflammatory factors (including TNF-a, CCL-2, CXCL1, and IL-1b) and increased insulin receptor substrate 1 (IRS-1) expression, which finally increased the insulin sensitivity in T2D model mice. Researchers also used CLAN to co-deliver Cas9 mRNA and sgRNA for the treatment of inflammatory diseases by targeting the NLRP3 inflammasome via CRISPR-Cas9 gene disruption (Figure 9B) [122]. The NLRP3 inflammasome has been reported to be a well-defined target for multiple NLRP3-dependent inflammatory diseases, for example attenuating septic shock, peritonitis, and T2D [127, 128]. Here researchers used CLAN to deliver Cas9 mRNA and sgNLRP3 into macrophages to NLRP3 gene disruption. Similarly, a library of CLANs was produced from PEG5K-PLGA11K, PLGA8K, and BHEM-Chol at different wt/wt ratios, and CLAN42, with the ratio 21.9:2.1:3 was screened to be the most preferable NP for mCas9/sgRNA delivery (abbreviated as CLANmCas9/sgNLRP3). In vitro, Cas9 protein could be expressed within 12h or 24h post-CLANmCas9/sgNLRP3 treatment in bone marrow-derived macrophages (BMDMs). The indel frequency can reach 70 % in NLRP3 locus when the BMDMs co-incubated with CLANmCas9/sgNLRP3 at the Cas9 mRNA dosage of 2.6 nM for 24h. The secretion of pro-inflammatory cytokines IL-1β and IL-18 was subsequently reduced, but CLANmCas9/sgNLRP3 did not 100 % inactivate the NLRP3 inflammasome limited by the transfection efficiency. In vivo, CLANmCas9/sgNLRP3 also disrupted NLRP3 dose-dependently, with low off-target effects. Researchers tried to use CLANmCas9/sgNLRP3 to treat LPS-induced septic shock, MSU-induced peritonitis and high-fat diet induced T2D. As a result, the efficient delivery of mCas9/sgNLRP3 by CLAN could alleviate acute and chronic inflammatory diseases, indicated by the down-regulation of pro-inflammatory cytokines.
Recently, the CLANs were also used to deliver Cas9 mRNA and sgRNA into dendritic cells (DCs) to induce transplant tolerance by blocking CD40 to inactivate T cells (Figure 9C)[129]. CD40 contributes to the activation and maturation of DCs, disrupt the CD40 signal in DCs can alternatively mitigate transplant tolerance without causing immune compromise or infections [130, 131]. The CD40 can be effectively edited by CLAN which co-delivered mCas9 and sgRNA which targeting CD40 (abbreviated as CLANmCas9/sgCD40) both in vitro and in vivo. The CLANmCas9/sgCD40 appeared to be 100 nm in diameters and the surface charge was about 10 mV. By transporting mCas9 and gRNA into bone marrow-derived dendritic cells, the total gene editing efficiency of CLANmCas9/sgCD40 in the CD40 locus was identified to be 26%, which decreased the expression of CD40 protein. The total indel frequency of CLANmCas9/sgCD40 in vivo was 11% after intravenous injection, indicating the successful delivery of CLANmCas9/sgCD40 into DCs. In an acute graft rejection mouse model, CLANmCas9/sgCD40 can mitigate transplant rejection and prolonged the skin graft survival via inhibiting the presentation of allogeneic antigens without causing nephrotoxicity and hepatotoxicity.

To reduce the off-target effects of CRISPR-Cas9 systems, specific gene editing in targeted sites is an alternative strategy. Successful examples have proved the specific genomic editing driving capability of organism-specific promoters in CRISPR-Cas9 systems [132, 133]. Recently, researchers utilized CLANs to deliver the macrophage-specific promoter (CD68) driven Cas9 plasmids (pM458 and pM330) for specific gene editing in monocytes and macrophages (Figure 9D)[134]. Through tail intravenous injection, CLANpM330 and CLANpM458 can also be trapped into tissue cells, T cells, B cells and neutrophils, but gene editing in these cells cannot be turned on because of the absence of CD68 promoter. Ntn1 was a potential therapeutic target of T2D in macrophages. After encoding sgRNA which targeting Ntn1 (sgNtn1) into pM330, the CLANpM330/sgNtn1 was specifically capable of disrupting the Ntn1 gene in macrophages and their precursor monocytes in vivo and thereby reducing the netrin-1 expression, which further ameliorated the T2D symptoms. As for the CLANpM330/sgNtn1 formulation, the indel frequency at Ntn1 locus was 33% (at a plasmid dose of 1.0 nM) in vitro, and 10% (at a plasmid dose of 1 mg/kg) in vivo specifically in monocytes and macrophages. However, the indel frequency of CLANpM330/sgNtn1 in neutrophils are as low as 2% even at the plasmid dose of 2 mg/kg, demonstrating that CLANpM330/sgNtn1 can effectively avoid the off-target effect under the control of CD68. Researchers also showed that targeting Ntn1 via CLANpM330/sgNtn1 could be a potential strategy for T2D therapy. As a result, both glucose tolerance and insulin sensitivity can be remarkably improved via treatment with CLANpM330/sgNtn1 at the plasmid dose of 1 or 2 mg/kg.
Figure 9. PEG-PLGA lipid NPs for CRISPR-Cas9 delivery. (A) PEG-PLGA lipid NPs delivering pCas9/NE for neutrophil-related inflammation modulation. Reprinted with permission from Ref. [125]. Copyright (2018) Elsevier. (B) PEG-PLGA lipid NPs carrying Cas9 mRNA and sgNLRP3 to macrophages for the amelioration inflammatory diseases. Reprinted from Ref. [122]. Under a Creative Commons Attribution 4.0 International License (C) PEG-PLGA lipid NPs delivering mCas9/sgCD40 to reprogram DCs and induce transplantation tolerance. Reprinted with permission from Ref. [129]. Copyright (2019) Elsevier. (D) PEG-PLGA lipid NPs delivering CD68 promoter-driven CRISPR-Cas9 plasmids for specific gene editing in monocytes and macrophages. Adapted with permission from Ref. [134]. Copyright (2018) American Chemical Society.
3.3 PEI

Cationic polymers play promising roles in the non-viral delivery of CRISPR-Cas9 systems, particularly for the nucleic formats (plasmid, Cas9 mRNA and sgRNA). The cationic polymers mainly includes PEI, polyamidoamine (PAMAM), and so forth. Generally, cationic polymers condense negatively charged nucleic acids into nano-sized packages via electrostatic attraction, protecting them from degradation and thereby facilitating their intracellular and nuclear transport for genomic editing.

PEI is one of the most widely used non-viral carriers for gene delivery [135, 136]. Because PEI has abundant amine groups, it can electrostatically interact with nucleic acids to form nanocomplexes. The PEI/nucleic acid nanocomplexes mainly enter cells via clathrin-mediated endocytosis pathway, and further get access to cytoplasm via the proton sponge effect [137, 138]. The major concern for PEI-based carriers entering clinic primarily is high cytotoxicity [42, 139, 140]. The cytotoxicity of PEI is closely related to its molecular weight [141]. Researchers employed BPEI\textsubscript{2K} to covalently modify Cas9 protein (SpCas9-bPEI), the SpCas9-bPEI was further mixed with sgRNA and packed into nanosized complex (Cr\textsubscript{-}Nanocomplex) [142]. The Cr\textsubscript{-}Nanocomplex transported effectively cross the thick bacterial cell wall of Methicillin-resistant \textit{Staphylococcus aureus} (MRSA), thereby liberated Cas9 proteins to induce DSB for gene editing. Under the guidance of sgRNA targeting the major resistance gene, mecA, the Cr\textsubscript{-}Nanocomplex can interfere the bacterial growth efficiently, showing the potential capability of Cr\textsubscript{-}Nanocomplex to be a target-specific antimicrobial agent.

To maximize delivery efficiency and minimize cytotoxicity of PEI-based delivery systems, researchers also applied post-modification methods to enhance transfection efficiency as well as reduce the cytotoxicity of PEI. For example, PEI-β-cyclodextrin (PC), a cationic polymer synthesized via carbonyldiimidazole chemistry between high-molecular-weight PEI (25 kDa) and β-cyclodextrin, was applied to deliver pDNA encoding Cas9/sgRNA into Hela cell lines for gene editing (\textbf{Figure 10A}) [143]. The PC was previously employed in the delivery of pDNA and siRNA both \textit{in vitro} and \textit{in vivo} for cancer therapy [144, 145]. By utilizing the PC to deliver CRISPR-Cas9 pDNA, the gene editing efficiency increased from 3 \% (using bare PEI) to 19 \% in hemoglobin subunit beta (HBB) locus and to 7 \% in rhomboid 5 homolog 1 (RHBDF1) locus.

PEG-PEI-Cholesterol (PPC) is a PEI-based lipopolymer that has been demonstrated to be a safe non-viral vector in phase II trials, albeit not yet used in the delivery of CRISPR-Cas9 cargos [146]. Recently, the PPC was further formulated with osteosarcoma (OS)-specific aptamer LC09 (LC09-PEG2000-DSPE) to deliver CRISPR-Cas9 pDNA encoding vascular endothelial growth factor A (VEGFA) sgRNA and Cas9 for effective VEGFA genome editing \textit{in vivo} (\textbf{Figure 10B}) [147, 148]. The molecular weight of BPEI used here is 1800 Da. The LC09-PPC-CRISPR-Cas9 had a diameter of 163.1 nm and a zeta potential of -12.7 mV. With the help of LC09, the LC09-PPC-CRISPR/Cas9 internalized into OS cells (K7M2) more efficiently via micropinocytosis than that of Lipofectamine 3000-CRISPR-Cas9. LC09-PPC-CRISPR-Cas9 had the capability of inhibiting the
growth, migration and invasion of K7M2 by causing high indel frequency. *In vivo* biodistribution study further proved that LC09-PPC-CRISPR-Cas9 had the target ability against K7M2 both in orthotopic and metastatic models, which paved the way for targeted cancer therapy. After the intravenous injection of LC09-PPC-CRISPR-Cas9 (at the plasmid concentration of 0.75 mg/kg) three times, significantly higher indel in VEGFA locus was induced in tumor sites, which further reduced VEGFA expression and thereby inhibiting the tumor malignancy and metastasis. More importantly, reduced angiogenesis and bone lesion without toxicity were observed, which demonstrated the safety of LC09-PPC systems.

**Figure 10.** PEI based non-viral vectors for the delivery of CRISPR-Cas9 tools. (A) Schematic illustration of PC-mediated Cas9/sgRNA plasmid delivery for genome editing. Reprinted with permission from Ref. [143]. Copyright (2018) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (B) The schematic diagram utilizing lipopolymer PEG-PEI-Cholesterol to encapsulate CRISPR-Cas9 plasmids and to anchor LC09 aptamer for genome editing. Reprinted with permission from Ref. [148]. Copyright (2017) Elsevier.

3. 4 PAMAM

PAMAM dendrimers are another class of commonly used non-viral vectors for bio-therapeutics delivery, for example gene and proteins [149]. PAMAM has abundant primary amines on the periphery and tertiary amines in the interior.
Peripheral primary amines provide high positive charges to electronically interact with anionic matters, while interior tertiary amines facilitate the endosomal escape after intracellular transport.

To present the relationship between physical flexibility and efficiency of pDNA delivery, researchers synthetically engineered PAMAM into dendronized polymers and rationally regulated the flexibility by varying the dendron generation and density (Figure 11A) [150]. For the delivery of EGFP-encoding small pDNA (5.3 kb) into hard-to-transfect MCF-7 cell lines, the polymer 10c with 17 % anchoring ratio of generation 5 (G5) PAMAM dendron exhibited the most superior transfection efficiency without causing cytotoxicity. Such results revealed that conformational flexibility, determined by dendron generation and density on PHEMA-ran-PGMA, strongly influenced transfection efficiency. The researchers also fluorinated G5-dendron-substituted polymers to further improve efficiency of delivery via increasing cellular uptake and improving endosomal escape. After fluorination, the fluorinated 12c had higher transfection efficiency than that of commercial Lipofectamine 2000 when delivering a large EGFP-expressing plasmid (10.3 kb) into MCF-7 cells. More importantly, the polymer 12c achieved higher mammalian serine protease inhibitor expression in MCF-7 cell lines at both mRNA and protein levels than Lipofectamine 2000 in delivering CRISPR-dCas9 plasmids.

Using PAMAM to deliver Cas9 protein into the cytoplasm remains to be a challenge. However, guanidinobenzoic acid conjugated G5 PAMAM dendrimer can achieve intracellular delivery of proteins because it simultaneously provided the cationic dendrimer skeleton, membrane-disruptive region and multivalent protein binding units [151]. Inspired by this, researchers proved that PBA modified PAMAM had robust and unprecedented efficiency of delivery of 13 cargo proteins with different isoelectric points, which included the Cas9 RNP (Figure 11B) [152]. The PBA structure provided a combined effect of nitrogen-borinate coordination and cationic-π interaction between proteins and PBA-modified PAMAM. The PAMAM which conjugated 60 PBAs on the surface (P4) exhibited the highest transduction efficiency. The complex of P4/RNP turned out to be spherical NPs with around 300 nm in diameter. The P4/RNP treated group caused 40 % EGFP loss in 293T-EGFP cells, which was higher than that of CRISPRMAX, a commercialized RNP delivery agent. P4/RNP can also effectively realize gene editing of AAVS1 and HBB locus in 293T cells with the indel rates respectively to be 23 % and 21 %. Further, P4/RNP also rendered the editing of the catenin beta-1 (CTNNB1) gene in HCT-116 and HT-29 cell lines, demonstrated the universality of P4. However, owing to the large size of P4/RNP, P4 seems not suitable for RNP in vivo delivery, and further modifications to meet in vivo specified genomic editing are still needed. We think that making the PAMAM-based systems into multi-layer NPs may be a potential way to enhance their in vivo performance.
Figure 11. PAMAM based delivery systems for CRISPR-Cas9 tools. (A) Systematic dendronized polymer design for flexibility alteration by varying dendron generation and dendron density and their transfection efficiency in MCF-7 cells. Adapted from Ref. [150], Copyright (2017) The Royal Society of Chemistry. (B) Mechanism of boronate-rich PAMAM dendrimer in complexation with protein and its mediated Cas9/sgEGFP delivery for efficient EGFP genome editing. Reprinted from Ref. [152], Copyright (2019) The Authors, some rights reserved. Exclusive licensee American Association for the Advancement of Science. Distributed under a Creative Commons Attribution NonCommercial License 4.0.

3.5 Zwitterionic NPs

Zwitterionic NPs are well known for delivery applications because of their excellent stealth properties which can resist the formation of protein corona in the bloodstream, thereby significantly elongate the circulation time [153-155]. Recently, zwitterionic amino lipids (ZAL) has been reported to be suitable non-viral vectors for simultaneously delivering long Cas9 mRNA and sgRNA into a single NP both in vitro and in vivo (Figure 12) [156]. The improved long RNA delivery capability of ZAL can be achieved by tuning the structures of zwitterionic and cationic lipids. The zwitterionic sulfobetaine head groups and hydrophobic tails were connected by amine-rich linkers, and ZA3-Ep10 was screened out to be the most efficacious formulation for the delivery of Cas9 mRNA and sgRNA. In vitro, the Cas9 mRNA can enter into A519 lung cancer cells within 4 h co-incubation by ZA3-Ep10, followed by the translation of mRNA and the highest Cas9 protein expression can be achieved at 36 h. When the encapsulation ratio of Cas9 mRNA:sgRNA into ZA3-Ep10 was higher than or equal to 3:1 (wt), the gene editing efficiency accordingly became much higher. In vivo, ZA3-Ep10 encapsulating Cas9-mRNA and sgLoxP (4:1, wt) at 5 mg/kg total RNA was intravenously administered into the
engineered mice which containing Lox-Stop-Lox tdTomato (tdTO) cassette for stop cassette deletion and tdTO expression [157]. The tdTO fluorescent signals can be clearly detected in liver, lung and kidney one week post injection, proving the successful in vivo genomic editing. The systematic toxicity was not observed after the injection.

Figure 12. Chemical structures of ZALs that designed to increase molecular interactions with longer RNAs. Reprinted with permission from Ref. [156]. Copyright (2017) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

3.6 Two-dimensional (2D) materials

2D materials, for example graphene oxide (GO) and black phosphorus nanosheets (BPs), have attracted tremendous interests in biomedical applications and are recently utilized to deliver CRISPR-Cas9 systems [158, 159]. The planar structure of these 2D materials gives rise to high surface area, which further enhances the payload capacity [160, 161]. The PEG and PEI dual-functionalized GO was applied to deliver RNP via both electrostatic and π-π interactions (GO-PEG-PEI/Cas9/sgRNA) for gene editing in AGS-EGFP cells (Figure 13A) [158]. The GO-PEG-PEI/Cas9/sgRNA was approximately 9 nm in thickness and the rigid GO plane can efficiently shield RNP from enzymatic degradation. When incubated with AGS-EGFP cells, a high gene disruption level of 39% in EGFP locus was observed while the GO-PEG-PEI/Cas9/sgRNA exhibited low toxicity. This work proved the potentials of 2D materials in delivering CRISPR-Cas9 RNP [158]. Recently, researchers used the 2D biodegradable BPs to load RNP, enhancing its cytosolic delivery and release for genome editing (Figure 13B) [159]. The Cas9 protein was engineered with three
repeats of NLSs (Cas9N3) to improve nuclear targeting ability, meanwhile, the positive charge helped to electrostatically interact with anionic BPs. The BP loading capacity of Cas9N3 was remarkably high (99 %). The Cas9N3-BPs entered the cells mainly through direct membrane penetration and endocytosis pathway. Along with the intracellular degradation of BPs, the Cas9N3 can be released followed by nuclear localization under the guidance of NLSs, thus resulting in highly efficient genome editing. *In vitro*, Cas9N3-BPs exhibited outperformed indel frequency in different cell lines and locuses. The editing efficacy in Target 1 and GRIN2B was 27 % and 32 % in MCF-7 cell lines, and that in Target 1 and GRIN2B was 27 % and 23 % in human bone marrow derived mesenchymal stem cells. The Cas9N3-BPs also induced higher transfection efficiency (18 %) than that of commercial Lipofectamine (8 %) in hard-to-transfect cells (RAW 264.7). Moreover, the Cas9N3-BPs can effectively induce EGFP knockdown in A549/EGFP tumor-bearing mice. Collectively, the Cas9N3-BPs provided a simple and versatile strategy for RNP cytosolic delivery. These two works inspired the potential uses of other 2D materials, such as molybdenum disulfide (MoS2) and manganese dioxide (MnO2), for gene editing tools delivery [162, 163].

Since undisputable progresses of utilizing 2D materials to deliver gene-editing components have been made, more challenges towards future *in vivo* applications should be critically considered. The preparation of 2D materials still needs to be further optimized, including reliable scale-up production of the 2D materials and surface modification methodologies. Besides, the *in vivo* biological behaviors and systemic toxicity assessment of 2D materials should be studied. Properties and toxicity profiles of 2D materials are fundamentally determined by their characteristics, for example thickness, size and crystal phase. However, the relationship study between 2D materials and their *in vivo* metabolism has rarely been reported. Although there are some studies to claim the biodegradation capability of kinds of 2D materials (for example MoS2 and BP), more in-depth systematic and long-term evaluation on the biosafety issues are still required.
3.7 Metal-organic frameworks (MOF)

MOF is an emerging class of porous crystalline nanomaterials derived from the self-assembly between metal ions and organic linkers. By incorporating different building blocks, the functionalities of MOFs can be well tuned and optimized to expand their bio-applications including drug delivery [164, 165]. MOFs have the
capability of loading bio-therapeutics such as nucleic acid or protein because of their pore structures [166]. Recently, an ATP-responsive MOF constructed by zinc ions and zeolitic imidazole framework-90 (ZIF-90) was used to deliver Cas9 protein for genome editing (Figure 14) [167]. The ATP-triggered response mainly derived from the competitive coordination between ATP and Zn$^{2+}$ in the presence of ATP. The subsequent delivery of ZIF-90/Cas9 NPs and sgRNA$^{GFP}$ into Hela-GFP cells efficiently induced 40 % GFP protein knockout, which indicated the successful protein delivery. Considering the commonly upregulated ATP concentration in disease cells, such ATP-responsive delivery systems open up new avenues for CRISPR-Cas9 based disease therapy [168]. In another study, nanoscale ZIF-8, a subclass of MOF constructed by zinc ions and 2-methylimidazole was a high efficiency non-viral vector for RNP [169]. The RNP loading efficiency of ZIF-8 was 17 % (CC-ZIFs). The CC-ZIFs was about 100 nm in diameter with the surface charge at 5 mV. Moreover, the release of RNP from CC-ZIFs was a pH responsive mechanism, that is, more than 60 % proteins can be released within 10 min at lower pH (5 or 6) while merely less than 3 % proteins released under pH=7. In vitro, the imidazole ring in CC-ZIFs assisted its fast endosome escape, which benefited the following nucleus transport. The CC-ZIFs can effectively knock-out 37 % GFP proteins, which was superior to that of commercial Lipofectamine CRISPRMAX Cas9. Cancer cell membrane coating has been a straightforward strategy to enable the target and cell specific delivery of therapeutic cargos. Recently, researchers used the membrane of human breast adenocarcinoma cells (MCF-7) to coat CC-ZIF, which resulted in biomimetic core-shell NPs (C$^{3}$-ZIF$^{MCF}$) with an average size of 120 nm [170]. The inherent homotypic binding phenomenon greatly enhanced the efficiency of specific delivery and genome editing. In vitro, MCF-7 cells exhibited the highest uptake of C$^{3}$-ZIF$^{MCF}$ compared to other cell types. As a result, C$^{3}$-ZIF$^{MCF}$ knocked down 3-fold expression of EGFP while C$^{3}$-ZIF$^{HELA}$ knocked down 1-fold of that in MCF-7 cells. In vivo, the C$^{3}$-ZIF$^{MCF}$ can selectively transport the RNP to MCF-7 tumor cells in a xenograft model. This work demonstrated the potential of expanding the delivery applications of MOFs by taking them as building blocks of multilayer NPs.

Huge surface area, high porosity, tunable chemical compositions, tailored morphology, and controlled degradability have driven MOFs towards applications of drug delivery. As to the delivery of genome editing machinery, MOFs based non-viral vectors still face critical challenges towards their in vivo applications. First, the determination of the toxicity of MOFs is the most urgent issue to be solved towards in vivo research. Long-term toxicity of various MOFs appears to be unclear and requires extensive in vivo assessment. Besides, the maintenance of colloidal stability of MOFs in physiochemical conditions should be emphasized. Through improving the stability of MOFs, the toxic side effects which induced by the aggregation of MOFs can effectively decreased. Finally, in vivo fate and degradation mechanisms of various MOFs should also be comprehensively addressed.
4. Conclusions and perspective

Along with the approval of about eight gene therapies worldwide since 2017, we are witnessing a boom of gene-based therapeutics [171-173]. The CRISPR-Cas9 system, a revolutionary gene manipulation technology that theoretically can edit target genes at almost any sequence, has provided unparalleled choices and opportunities in genetic disorder-induced diseases, for instance cancers, cardiovascular diseases, liver-related diseases, neurodegenerative diseases, inflammations, and even rare genetic diseases like hereditary tyrosinemia and LCA10 [50, 174-176].

The power of CRISPR-Cas9 critically depends on the successful delivery of gene editing cargos into the target cells. Viral vectors (such as LV) seems to be the limited choices for the delivery of CRISPR cargos in current CRISPR-based clinical trials. Generally, viral vectors exhibited very high efficiency in the delivery of CRISPR-Cas9 systems. We believe that there are inevitabilities between the high efficiency of delivery of viral vectors and their intrinsic structures. For example, the enveloped viruses (such as LV) usually exhibit multi-layer structures which includes nucleocapsid, viral tegument, envelope and envelop protein from the inside out. The multi-layer structures greatly shield and protect the CRISPR cargos from deactivation, and further ensure their transportation into host cells for genome editing. However, the intrinsic restrictions of viral vectors inevitably and severely hinder their further clinical translations. Particularly in terms of safety, viral vectors can induce serious damages to normal organs and cause systematical adverse effects because of the host-genome integration and immunogenicity. By contrast, non-viral delivery systems of CRISPR-Cas9 possess unique advantages such as: transient expression patterns, low immunogenicity, and feasibility of mass-production [31]. The development of non-viral delivery systems should be taken as important as the exploitation of CRISPR-based biological systems. Although the future of non-viral vectors is
promising, many obstacles still exist. Interestingly, the currently reported artificial multi-layer NPs exhibited considerable efficiency of delivery of CRISPR-Cas9 systems, demonstrating the superiorities of multi-layer structures in delivering CRISPR cargos. The multi-layer structures of these NPs are quite similar with that of viral vectors, for example: (1) a cationic core (organic or inorganic one) to complex and stabilize the CRISPR cargos; (2) an organic shell (mainly lipid layer) to further protect the inner complexes; (3) multifunctional ligands that anchor onto the shell to facilitate the bio-interactions with host cells. The CRISPR cargos can be efficiently and stably encapsulated into the multi-layer NPs, and the material compositions can significantly influence the extra/intra-cellular fate of the cargos, thereby the genome editing efficiency. To transport CRISPR tools into nucleus to exert their biological functions, an ideal non-viral vector needs to load the cargos inside efficiently, then accurately deliver the cargos to the specific site and further mediate the efficient cellular endocytosis of cargos. The carrier also needs to help the cargos to break through the intracellular barriers as well as to release the cargos timely and facilitate their nucleus entrance. Of course, the stability and bio-activity of the cargos must be ensured throughout the processes. We think that this can be achieved by reasonably selecting the non-viral materials and designing the structure of the multi-layer NPs. It also reminds us the possibilities that converting the currently potential building blocks into multi-layer NPs while maintaining their own delivery capabilities.

Two key words for the development of multi-layer NPs based CRISPR-Cas9 delivery are efficiency and safety. From the aspect of efficiency, the targeted delivery into intended tissues and specific cells should both be included. The biggest challenge is to specifically and efficiently deliver cargos into target sites by multi-layer NPs, which is quite crucial for successful clinical transformation. In this regard, the recent reported triple-targeting strategy may provide a feasible approach to enhance the efficiency of target delivery in vivo [50]. However, even higher efficient genome editing and therapeutic effect in vivo can be achieved by active targeting approaches, multi-layer NPs may encounter the same dilemma as most nanomedicines, for instance the protein corona formation and non-specific uptake by the reticular endothelial system [93, 177]. Thus, more in-depth understanding of nano-bio interactions are still needed, for instance: (1) the interaction and cause of nano-systems being taken up by mononuclear macrophage systems, (2) the dynamic mechanism between non-viral vectors and targeting sites. Secondly, the CRISPR-Cas9 tools must be accurately delivered into the nucleus where the gene editing takes place. After the specific localization at target sites, cellular uptake, endosomal escape, cytoplasmic transport, and nuclear import are necessary steps involved for the successful delivery, which is much more complex and difficult than traditional nucleic acid delivery. The positive surface charges of commonly used vectors may facilitate the cellular uptake of NPs, but the specificity need to be cautiously considered. Incorporating targeting molecules, antibodies, or aptamers onto the surface of NPs seem to be a feasible approach to improve specificity. But modifying the NPs with targeting moieties also increases the difficulties in incorporating additional components inside the vectors [174]. Thirdly, the efficient cargo release inside cells in
a spatiotemporal manner is another important issue. Otherwise, the gene editing tools would not work at a specific time window, which can result in unexpected off-target effects. Effective strategies such as controlling the activity of Cas9 protein with a small molecule has been established [26]. Researchers used disulfide linkages contained materials to build delivery systems and achieved GSH triggered release of gene editing tools, which further enhanced the editing efficiency [111]. Moreover, we fully utilized the photo-thermal conversion abilities of gold NPs to achieve the intracellular thermos-triggered plasmid release under the stimulation of external laser [76]. These methods offer approaches to spatiotemporally control the cargo release, and further improve the efficiency and accuracy of CRISPR/Cas9 systems by optimization of the applied materials. Following by the cargo release, the CRISPR-Cas9 system can be imported into the nuclei under the guidance of incorporated nucleus targeting ligands (such as TAT, NLS).

In most cases, cationic materials are utilized to build multi-layer NPs for the delivery of CRISPR-Cas9 systems, thus the safety becomes another major concern. Cationic materials possess some intrinsic drawbacks as delivery vectors. (1) The cationic components easily disturb and destabilize the cell membrane, which further induce serious cytotoxicity. (2) Cationic vectors, for example cationic liposomes, mainly internalized into cells via endocytosis, followed by entrapping in endosomes or lysosomes. But the acid environment easily degrades cargos and thereby reduce transfection efficiency. (3) Mononuclear phagocytic system acts as scavenger of the cationic systems during the blood circulation, which significantly hinders the accuracy of cargo transportation. Recently, the successful employment of non-cationic materials for in vivo CRISPR-Cas9 based gene therapy of TNBC offers an alternative strategy to cationic materials [93]. Moreover, the biodegradability of used materials should be valued [92, 178]. Utilizing FDA-approved pharmaceutical excipients as the building components may be a feasible way to design and construct the delivery systems. The particular advantage is that their metabolic products have been well studied, and their metabolic pathways are clear, which ensures the biocompatibility and biosafety. This approach may greatly shorten the development cycle and accelerate the clinical application. On the other hand, the CRISPR-Cas9 system itself has the risk of off-target effect, which means the unexpected editing in the genome of targeted cells. To alleviate this, rationally designing the sgRNA and deliberately choosing the targeting site seem to be effective approaches. Moreover, Cas9 mRNA and protein formats are superior to Cas9 plasmid from the prospective of lowering off-target effect, which may induce exhibit higher efficiency of DSB formation with the help of non-viral carriers.

The CRISPR-Cas9 based therapies progressed rapidly and remarkably in past decade and some of them have already entered into clinical trials. We are welcoming the revolutionary future that utilizing gene editing technologies to treat diseases. Meanwhile, we are quite optimistic that the multi-layer NPs, which have structures similar to viral vectors, will further help us to broaden implementations of genome editing technologies, especially for therapeutic bio-applications in vivo. In the near future, this “magic scissor” technology will offer new therapeutic approaches for
human diseases that currently unable to treat.

5. Glossary

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NGF  Nerve Growth Factor
NLS  Nuclear Localization Signal
PEG-PLGA  Poly (Ethylene Glycol)-Poly (Lactic-co-Glycolic Acid)
PEI  Polyethylenimine
PAMAM  Polyamidoamine
Plk1  Polo-Like Kinase-1
RNP  Ribonucleoprotein
sgRNA  Single Guide RNA
TALENs  Transcription Activator-Like Effectors Nucleases
TAT peptide  HIV-1-Transactivator of Transcription Peptide
TNBC  Triple-Negative Breast Cancer
T2D  Type 2 Diabetes
UCNPs  Up-conversion Nanoparticles
ZFNs  Zinc Finger Nucleases
2D  Two-Dimensional

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Declarations of interest: none.

References
[5] B.L. Stoddard, Homing endonucleases: from microbial genetic invaders to


genome editing based on a polymer-derivatized CRISPR nanocomplex for targeting bacterial pathogens and antibiotic resistance, Bioconjugate Chem. 28 (2017) 957-967.


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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>PEG-PLGA</td>
<td>Poly (Ethylene Glycol)-Poly (Lactic-co-Glycolic Acid)</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Polyamidoamine</td>
</tr>
<tr>
<td>Plk1</td>
<td>Polo-Like Kinase-1</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>sgRNA</td>
<td>Single Guide RNA</td>
</tr>
<tr>
<td>TALENs</td>
<td>Transcription Activator-Like Effector Nucleases</td>
</tr>
<tr>
<td>TAT peptide</td>
<td>HIV-1-Transactivator of Transcription Peptide</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple-Negative Breast Cancer</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
</tr>
<tr>
<td>UCNPs</td>
<td>Up-conversion Nanoparticles</td>
</tr>
<tr>
<td>ZFNs</td>
<td>Zinc-Finger Nucleases</td>
</tr>
<tr>
<td>2D</td>
<td>Two-Dimensional</td>
</tr>
<tr>
<td>Intervention/Treatment</td>
<td>Edited Cells</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Genetic: Edited T Cells</td>
<td>T cells</td>
</tr>
<tr>
<td>Drug: CTX</td>
<td></td>
</tr>
<tr>
<td>Edited CD34+ HSPCs</td>
<td>CD34+ HSPCs</td>
</tr>
<tr>
<td>TALEN-HPV E6/E7</td>
<td>HPV16 and HPV18</td>
</tr>
<tr>
<td>CRISPR-Cas9-HPV E6/E7</td>
<td></td>
</tr>
<tr>
<td>UCART019</td>
<td>CAR-T cells</td>
</tr>
<tr>
<td>Genetic: Edited EBV-CTL cells</td>
<td>EBV-CTL cells</td>
</tr>
<tr>
<td>Drug: FA/CTX/IL-2</td>
<td></td>
</tr>
<tr>
<td>Genetic: NY-ESO-1 redirected autologous T cells</td>
<td>T cells</td>
</tr>
<tr>
<td>Drug: CTX/FA</td>
<td></td>
</tr>
<tr>
<td>anti-MSLN CAR-T cells</td>
<td>CAR-T cells</td>
</tr>
<tr>
<td>Genetic: MSLN-directed CAR-T cells</td>
<td>CAR-T cells</td>
</tr>
<tr>
<td>Drug: PTX/CTX</td>
<td></td>
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<tr>
<td>CTX001</td>
<td>CD34+ hHSPCs</td>
</tr>
<tr>
<td>Edited Patient-specific iHSCs</td>
<td>iHSCs</td>
</tr>
<tr>
<td>Universal Dual Specificity CD19 and CD20 or CD22</td>
<td>CAR-T Cells</td>
</tr>
<tr>
<td>CAR-T Cells</td>
<td>or</td>
</tr>
<tr>
<td>-------------</td>
<td>----</td>
</tr>
<tr>
<td>Genetic: XYF19</td>
<td>CD19+</td>
</tr>
<tr>
<td>CAR-T cells</td>
<td>CAR-T cells</td>
</tr>
<tr>
<td>Drug: CTX/FA</td>
<td>HPKI</td>
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<tr>
<td>CTX110</td>
<td>T cells</td>
</tr>
<tr>
<td>AGN-151587 (EDIT-101)</td>
<td>Photoreceptor or cells</td>
</tr>
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