Chapter 12
Novel Nanotechnology-Based Vector Delivery in CRISPR System for Transgene-Free Editing

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12.1 Introduction

CRISPR (clustered regularly short palindromic repeats)/Cas (CRISPR-associated proteins) is an acquired and advanced phage immune system discovered in many arches and bacteria to protect them against invasive genetic materials such as nucleic acid (Ahmar et al. 2020; Barrangou et al. 2007; Fineran and Charpentier 2012; Horvath and Barrangou 2010; Wiedenheft et al. 2012). Since its first application in 2013, it was first introduced in mammalian cells. CRISPR mechanism is based on RNA-guided programmed nuclease, which has altered much more functions than just editing of genome (Cong et al. 2013; Mali et al. 2013). The CRISPR/Cas technology is based on functionalized target sequences, core components, and multiple subunits that have been characterized into three classes and their subtypes (Gasiunas and Siksnys 2013; Makarova et al. 2011a, b). The Type I CRISPR systems consisted of CRISPR-associated complex for antiviral defense (CASCADE) structure that comprised various subunits of Cas proteins that bind and form complexes with CRISPR RNA (crRNA) to initiate and signal the desired target loci. However, in Type III, crRNAs are integrated into various subunits complex called Csm or Cmr to signal, bind, and cleave the invaded RNA. On the contrary, in Type II systems, the Cas9 protein is the only prerequisite for the integration of DNA (Brouns et al. 2008; Garneau et al. 2010; Hale et al. 2009; Hsu et al. 2014). The Type II Cas system from specie *Streptococcus pyogenes* has a broad-term application in biomedical research due to the processive features such as high efficiency, specificity, rapid, inexpensive, simplicity as well as great versatility (Bikard et al. 2014; Cho et al. 2013; Hwang et al. 2013; Nekrasov et al. 2013; Wu et al. 2015). This system consists of two functional components: guide RNA (gRNA) and a DNA endonuclease (Cas9), which were engineered by the fusion of tracrRNA and a crRNA into a single RNA molecule. Usually, gRNA is easily switched by a synthetic single-guide RNA (sgRNA). Target site recognition begins in the presence of protospacer-adjacent motif (PAM) that is immediately located at (50-NGG) site, where the Cas9 signals the sgRNA to be positioned to the targeted site to initiate and unwind the site-specific double-stranded DNA breaks (DSBs); in these cases, the two cellular repair pathways, homology-directed repair (HDR) and nonhomologous end joining (NHEJ), can ensure to initiate with the potential alterations or error-prone insertions/deletions (indel) products (Garneau et al. 2010; Gasiunas et al. 2012; Jinek et al. 2012; Sapranauskas et al. 2011). Furthermore, in recent years, CRISPR interference (CRISPRi) technology has been developed that utilized deactivated dead Cas9 (dCas9) protein showing absence or no endonuclease activity, thus actively regulating the genes in a well-defined manner (Gilbert et al. 2013; Larson et al. 2013; Qi et al. 2013). In addition, the Cas9 nickases (HNHH840A or RuvCD10A), which cleave only a single strand other than both strands of the target site, have become effective for genome editing mechanism (Guilinger et al. 2014; Ran et al. 2013a). Due to its high versatility, this mechanism is much appropriate for the editing of the genome with less off-target effects in any model organism (Kleinstiver et al. 2016; Shen et al. 2014). More recently, an alternate technology based on CRISPR-nuclease

Cpf1 has been efficiently developed that showed various advantages as well as disadvantages of each Cas9 system (Zetsche et al. 2015, 2017).

Presently, the delivery approaches of the CRISPR/Cas system are primarily focused on physical strategies (transformation, electroporation, microinjection, and so on) via viral and/or nonviral vectors (Adeno-associated virus (AAV), lentivirus, adenovirus), etc. (Niu et al. 2014; Xue et al. 2014; Yu et al. 2017). In the above-mentioned platforms, the physical and viral vectors have been subjected to be achieved through powerful delivering components of CRISPR/Cas9 systems. Despite high editing efficiencies, most of the physical delivery methods are only applicable for in vitro but not fit for in vivo applications (Chen et al. 2017). For viral vectors, the potential apprehensions are the limited DNA packaging capacity, limited scale-up production rate as well as clinical therapeutics such as carcinogenesis and immunogenicity (Chen and Gonçalves 2016). Nonetheless, the current delivery strategy for nonviral-based nanoparticles showed its significant considerations to overcome the limitations of safety concerns (Mintzer and Simanek 2009; Pack et al. 2005).

The emergence of material sciences and nanotechnology has offered tunable and significant aspects that hold potent applications in the field of genome editing (Yin et al. 2017). However, lipid and polymeric-based nanoparticles (NPs) offered encapsulation of large size genetic payloads and favor high efficiency and immunogenicity response (Liu et al. 2018a, b). However, gold-based nanoparticles (AuNPs) have triggered the delivering approach of ribonucleoprotein (RNP) in vitro and in vivo applications in mice (Lee et al. 2017). Up to date, previous pieces of literature focused on nonviral NPs-based delivery in the CRISPR system, which aims to enhance the delivery efficiencies, mitigate the off-target effects, and recognize Cas9 protein on the target sites (Dever et al. 2016; Yin et al. 2016). This chapter exclusively elaborates the recent barriers for the delivery of the CRISPR/Cas9 system with great emphasis on the potential development of vector delivery in CRISPR/Cas9 based on nanomaterials for transgene-free genome editing applications.

12.2 Limitations and Challenges of CRISPR/Cas9-Based Genome Editing

So many technical pitfalls need to be addressed before CRISPR/Cas genome editing system can be efficiently utilized for clinical use. Based on a genetic basis, three consecutive limitations are subjected to be addressed. First, signaling and targeting the desired site actively, accurately, and efficiently of both cleavage and repair machinery to mitigate the chances of off-target possibility. Second, consider switching repair pathways such as HDR- or-NHEJ that handicamp on various experimental designs. Third, the system requires to be more specific to trigger therapeutics applications in diagnosing multiple diseases. However, due to the high probability of infrequent recombination efficiency, there are various shortcomings to achieve

potential applications, as presented in Fig. 12.1 (Wang et al. 2013; Weber et al. 2015; Yang et al. 2013; Yu et al. 2015). Therefore, the gene-based delivery vehicle of CRISPR components governs the integration of foreign genes into the desired genomic site, and the resultant leads to poor immune feedback. However, various viral vectors are functionally utilized for in vitro delivery vehicles of CRISPR-based reagents, and the frequency of activation mutagenesis leads to the generation of proto-oncogenes due to the insertion of viral genes into the desired target genome, which results in the development of tumorigenesis (Yin et al. 2016). Recently, in vivo delivery of nano-based vectors in the CRISPR system could resolve these concerns to overcome these pitfalls for transgene-free editing.

12.3 Modes of CRISPR/Cas9 Delivery Approaches

Usually, sgRNA can be integrated into the vector plasmids (pX459, pX330, etc.) that consist of sgRNA complex or can be achieved through in vitro transcription. In contrast, the desired template single-stranded oligodeoxynucleotide (ssODN) required for the gene correction through HDR-mediated pathway can be constructed in plasmids or achieved through in vitro mechanism.

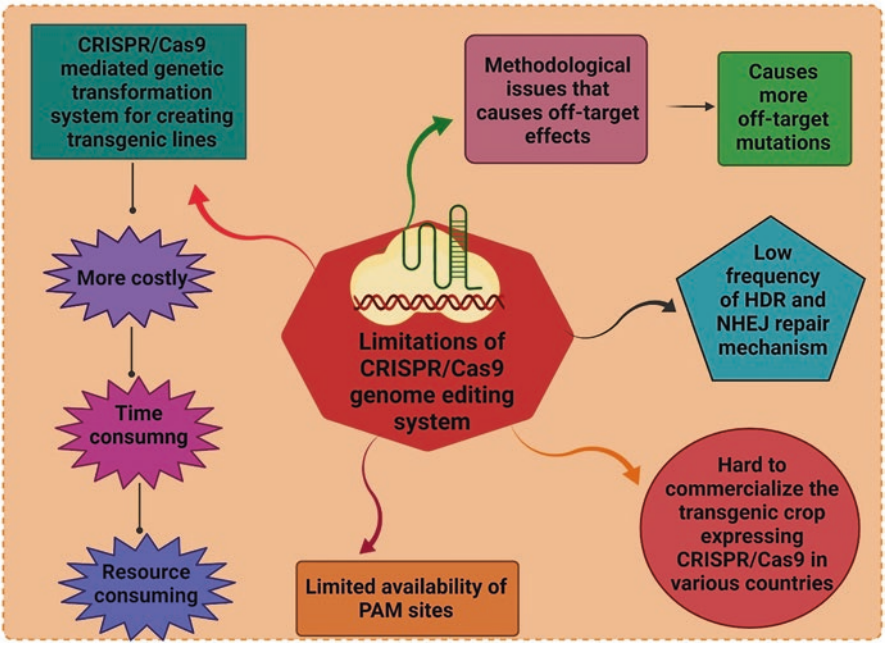

Fig. 12.1 Limitations and challenges of CRISPR/Cas9 genome editing system

Multiple CRISPR/Cas9 cargoes having different characteristic features can be integrated into various genetic payloads, such as DNA, mRNA, sgRNA, protein, and plasmids, respectively. Each of these genetic repertories encapsulated with NPs for vector delivering system showed pros and cons to fulfill the possessive challenges as summarized in Table 12.1.

The most forthcoming strategy is to integrate the Cas9 cassette into the sgRNA scaffold efficiently. Principally, this procedure triggers immediate gene editing as there are no chances of transcriptional and translational control. It also progressively offers transient gene expression with less toxicity and mitigates off-target effects. Moreover, the positively charged Cas9 protein, the negatively charged sgRNA, as well as large-sized Cas9 protein (~160 kD) can inhibit the direct and

Table 12.1 Various forms of CRISPR/Cas9 repertories and NPs for vector delivery system

CRISPR/Cas9 repertories	Nano-based delivering platforms	Advantages	Disadvantages
Cas9 protein, and sgRNA	Lipid NPs	Low toxicity, minimum off-target effects, poor stability, less inflammatory response (Chen et al. 2020) Commercially utilized cationic transfection lipids that form stables complexes with genetic payloads such as DNA, RNA, mRNA, and proteins, respectively (Chen et al. 2020) Significant scale-up and manufacturing efficiency (Evers et al. 2018)	High cost, low transfection efficiency, high encapsulation risk, rapidly degraded from the nucleases. Poor stability, endotoxin effect that leads to cellular toxicity (Li et al. 2018)
Cas9 mRNA and sgRNA	Polymeric NPs	A novel form of PACE-based cationic polymers showed higher transfection capacity (Wahane et al. 2020) Provide a broad-term application based on biocompatibility, biodegradability, temperature, light-sensitive, pH, and low immunological response, respectively (Wahane et al. 2020)	Difficulty in manufacturing large-scale efficiency, cellular toxicity, rapidly escape from the endosomal membrane during DNA repair mechanism (Wahane et al. 2020)
CRISPR/Cas9 plasmid	Gold NPs	High delivering, editing efficiency, and reduced off-target effects (Lee et al. 2017) The modified form of the CRISPR-gold RNP vector proposed a low toxic effect (Mout and Rotello 2017) Handy to maintain by the context of charge and size distribution (Chen et al. 2019)	Existence of cationic charge governs the speedy escape from the endosomal barrier (Deng et al. 2019)

efficient delivery of Cas9/sgRNA ribonucleoprotein (RNPs) cassettes (Subburaj et al. 2016; Zuris et al. 2015). On the contrary, the suitable and handy option is the interaction of Cas9/mRNA with sgRNA (Shen et al. 2014). Furthermore, the third choice is the encapsulation of plasmid-based CRISPR/Cas9 complexes (Ran et al. 2013b). Interestingly, this is an attractive delivering strategy due to its cost-effectiveness, efficiency, and simplicity. The Cas9 and sgRNA complexes, as well as the desired HDR template, can be easily integrated into the same plasmid, which reveals higher stability than mRNA and protein. However, the larger plasmid size (>7 kb), as well as Cas9 protein (~4.5 kb) potentially, enhances the limitations of delivery cargoes and the mechanism of CRISPR/Cas9 genetic payloads (Ran et al. 2015).

12.4 Recent Nano-Based Vector Delivery Modes for the CRISPR System

12.4.1 Viral and Nonviral Delivery Modes for the CRISPR System

The systematic CRISPR/Cas genome editing technique has rapidly expanded significant concerns in the area of biomedical research, especially for the treatment of genetic disorders and cancer therapeutics, respectively. The efficient delivery approaches showed a pivotal role in the applications of the CRISPR/Cas9 genome editing system. In recent years, both viral and nonviral vector approaches have been studied well for sgRNA delivery systems. Conventionally, the viral vectors consisting of lentivirus (Chakraborty et al. 2014) and adeno-associated virus (Long et al. 2016) have tremendously limited the capacity for the delivery of CRISPR/Cas components because of the generation of undesired mutations ratios, high off-target effects, integrational mutagenesis (Schumann et al. 2015), immunogenicity, limited packaging capacity, as well as carcinogenesis probability (Kay 2011). In contrast to viral vectors, the deliveries of nonviral vectors of the CRISPR/Cas system through nanoparticles (NPs) may significantly address various challenges recently. These recent challenges include safety issues (Schmidt and Grimm 2015), huge packaging of genetic payloads (Chamberlain et al. 2016), constructed protocols (Li et al. 2015), low cost, and robust scale-up production (Ramamoorth and Narvekar 2015), which may tremendously address the above-mentioned challenges. Furthermore, the nonviral vectors can be constructed to deliver the genetic cargoes to the desired cell lines. The delivery system can be widely categorized into lipids, polymers, and gold NPs, respectively, as demonstrated in Fig. 12.2. The delivery of nonviral vectors based on CRISPR systems in the form of DNA, mRNA, protein, and Cas9 plasmid together with sgRNA has governed efficient delivering strategies in CRISPR/Cas systems for the applications of genome editing.

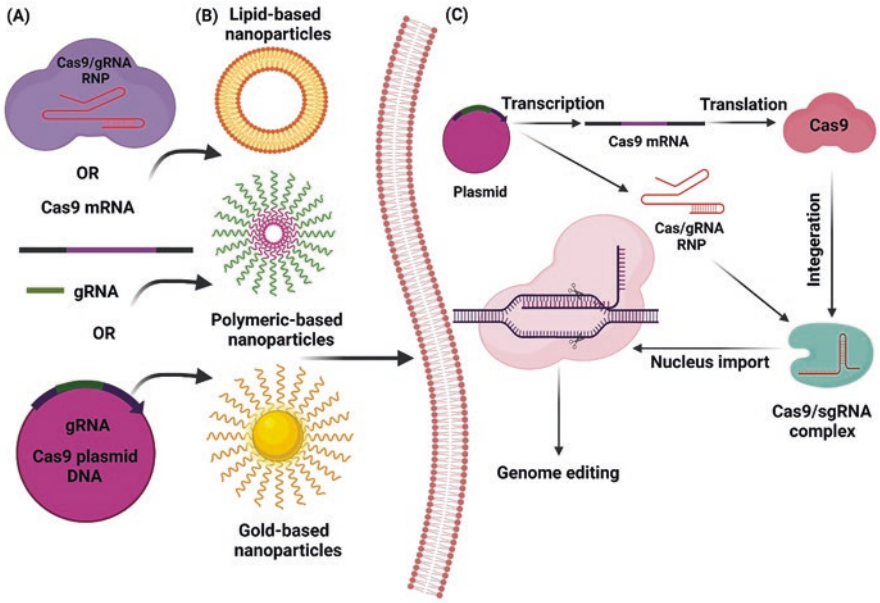

Fig. 12.2 Illustration of various NPs for the delivery of sgRNA and CRISPR/Cas system (**a**) Different strategies of CRISPR/Cas system. (**b**) Different types of NPs-based delivery systems for sgRNA and CRISPR/Cas system. (**c**) Genome editing of nanoparticles-based delivered Cas9/sgRNA plasmid DNA complex

12.4.2 Nonviral Delivery Modes of CRISPR/Cas9 System

12.4.2.1 Lipid NPs

Lipids hold the most remarkable and promising material for the potential delivery of nonviral-based genome editing systems (Mohammadinejad et al. 2020). The chemistry of various lipids is amphiphilic that possesses hydrophilic head and hydrophobic tails for the gene delivery system. More precisely, cationic lipid complexes progressively showed a helpful characteristic and have been considered adequate due to possessing charge-charge interaction with negatively charged DNA/RNP complexes. Previous studies literature proposed that these lipid-based complexes turn to decrease the chances of genetic cargoes by the degradation of nucleases (Möller et al. 2016). The ability of the most arsenal lipid-based nanoparticles (LNPs) is being the most versatile system for the delivery of genetic payloads and any other LNPs that have been an effective gene therapy for clinical researches (Felgner et al. 1987).

Appropriately, anionic charges present on DNA, mRNA, and gRNA are electrostatically attached with cationic charged lipids complexes to formulate LNPs (Cong et al. 2013). The lipid bilayer is not only helpful for the barrier of genetic payloads across the cell membrane barrier, but also hinders the genetic payloads from the

degradation of enzymatic, RNases, and immunological feedback (Liu et al. 2018b). Next, the context of commercially accessible lipids is a deep-rooted engineered delivery approach that was generated for CRISPR gRNA, mRNA, plasmids, and Cas9/sgRNA/RNPs complexes like RNAiMAX and lipofectamine that are utilized in combination with various cell lines for the treatment of gene therapeutics or gene knock-in/out in model organisms (Mout et al. 2017).

The positively charged Cas9 protein hampers the valuable characteristics of the commercially engineered cationic lipids owing to poor stability, high toxic effect, less inflammatory response, less transfection efficiency, and poor target delivery to the target sites. Though the engineered cationic lipids are used as a carrier in the CRISPR system, there are still some boundaries that hinder their further applications (Whitehead et al. 2009). Therefore, extensive alterations have been navigated for the modification of the above-mentioned pitfalls.

Engineered Cas9-sgRNA RNPs with Cas9 proteins forming complexes have been proposed to achieve 80% gene modification with the potential delivery achieved by LNPs in human cell lines (Zuris et al. 2015). Modified liposomes, such as 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol (Ch), and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), showed a great enhancement in cellular uptake efficiency and also modified pharmacokinetic distribution in vivo (Onuki et al. 2016). Engineered solid-LNPs can enhance the sustainability and storage stability for a wide range of applications for the lyophilization process (Leung et al. 2014). Previously reported literature ameliorated that the chemically modified lipids can progressively expand transfection efficiency and mitigate the toxic nature of cationic lipids (Kish et al. 2007) by structural modification of the ammonium groups via DOTAP (Wang et al. 2018), DC-Chol (Cardarelli et al. 2012), DOPE (Wang et al. 2018), as well as DOTMA (Lotti 2017), respectively. The first-generation liposomal-based vectors constructed through DOTMA were employed for the in vitro delivery of plasmid DNA. Moreover, DOTMA-based constructions mainly triggered cellular toxicity with the activation of immunological response owing to the presence of cationic charge. Therefore, DOTMA-based constructions cannot be applicable for in vivo studies. Additionally, LNPs are somehow distinct from liposomes due to the rapid integration with ionizable lipids and rapidly interact with genetic payloads such as nucleic acids and do not possess aqueous core components. Wheeler and his correspondence for the first time generated LNPs by utilizing stabilized plasmid particles (SPLPs) by following a detergent dialysis technique. Various synthetic lipids such as dioleoyl-ammonium chloride (DODAC) act as cationic lipids with a helper lipid 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE), and polyethylene glycol (PEG) was utilized for the encapsulation of plasmid cytomegalovirus chloramphenicol transference (pCMVCAT). The results showed that produced small particles own 70 nm is size to achieved encapsulation efficacy up to 70% (Wahane et al. 2020). Recently, LNPs are fabricated with different techniques such as microfluidic hydrodynamic focusing (MHF), T-junction mixing, and staggered herringbone mixing (SHM) consisting of ethanol that acts as a controller phase for the rapid solubilization of lipids (Evers et al. 2018). Furthermore, many lipids complexes have been synthesized to drive the efficient delivery of

mRNA, gRNA, plasmids, and Cas9-sgRNA RNPs complexes for various in vitro and in vivo applications (Chen et al. 2020).

12.4.2.2 Polymeric NPs

Polymeric vector is an extensively utilized nonviral strategy for the delivery of CRISPR cargoes which aims to drive the significance for the functionalization and chemical diversity for the CRISPR delivery applications with strong biodegradability, biocompatibility, and low immunological response. Recently reported studies concerning polymeric-based NPs, viz., polyethyleneimine (Givens et al. 2018), poly (L-lysine) (Spoelstra et al. 2021), chitosan (Qiao et al. 2019), and polyamidoamine (PAMAM) (Givens et al. 2018), have captivated a tremendous consideration for efficient delivery of genome editing. Usually, polymeric vectors can be constructed from chemical subunits, viz., chitosan and PLGA, with a wide range of applications. Like lipid carriers, polymeric carriers can diffuse across the cell membrane and defend the genetics cargoes from degradation pathways and immunological responses (Zou et al. 2016). Furthermore, owing to the flexible configuration of polymeric carriers, many polymeric carriers possess specific applications for a variety of in vitro and in vivo approaches such as: discharging of intracellular microenvironments, specificity for in vivo targeting of receptors across the cell membrane, and encapsulation of various genetic payloads (Chen et al. 2015). To overcome the toxicity issue, many scientists revealed that either formulation of cationic polymeric vectors by itself or with other lipids and polymers could mitigate the chances of toxicity (Zhang et al. 2019). Therefore, to overcome the pitfalls as mentioned above, next-generation cationic-based polymeric platforms such as poly[(2-dimethylamino) ethyl methacrylate] (pDMAEMA), biodegradable poly (B-amino ester) (PBAE) polymers, and PAMAM dendrimers were synthesized. Owing to consisting of tertiary amine groups, the synthesized pDMAEMA and PBAE polymers also assist in escaping from the endosomal layer and have proven to be of higher transfection efficacy (Wahane et al. 2020).

Scientists also confirmed that polymer-based NPs are prone to deliver CRISPR-mediated cargoes with superior editing efficiency for a desired target antimicrobial in contrast to lipids (Kang et al. 2017). Polymeric carriers were encapsulated with Cas9/sgRNA networks that facilitated delivery effectively into the human genome with low toxicity and higher editing efficiency (Sun et al. 2015). Previously reported studies suggested that PEI 25 kDa (BPEI-25K) is an efficient nonviral vehicle for in vitro CRISPR genome editing systems (Ryu et al. 2018). Subsequently, the amine-terminated polyamidation (PAMAM) dendrimer cationic polymer significantly triggered the potential delivery of intracellular cytosolic Cas9 proteins and achieved high genome editing in multiple cell lines (Liu et al. 2019). Therefore, researchers enhance the rate of transfection efficiency and reduce the chances of nontarget binding sites by synthesizing new and promising synthetic poly (amino-co-ester) (PACE)-based cationic polymers for the potential delivery of genetic payloads. The newly developed mechanism demonstrates a reduced cytotoxicity effect

in contrast to other forms of cationic-based polymers. The high molecular weight PACE system navigates higher transfection capacity owing to the formation of DNA systems as well as narrowing the range of genetic materials (Wahane et al. 2020).

12.4.2.3 Gold NPs

Gold nanoparticles (AuNPs) are extensively utilized for the CRISPR-mediated RNP delivery. The gRNA, Cas9 proteins, and AuNPs are co-encapsulated into synthesized NPs (Wang et al. 2018). Distinct from lipids, viruses, and polymer vectors, AuNPs are facile concerning charge distribution and size (Chen et al. 2019). In genome editing, the synthesized AuNPs demonstrated ~90% delivery efficacy and ~30% gene-editing potentiality for CRISPR/RNP-mediated strategy in multiple cell lines (Mout et al. 2017). Previously reported literature on mice assumed that AuNPs are attributed for both HDR in vivo and CRISPR gene editing through in vivo delivery of donor DNA and CRISPR RNP-mediated approach with reduced off-target effects and high editing efficiency in many cell lines (Lee et al. 2017). Scientists confirmed that the CRISPR-Au-RNP complex vector delivery system could edit various genes in the brains of multiple mice and showed no toxic effect (Mout and Rotello 2017). The CRISPR-Au complex was synthesized by AuNPs and co-assembled with poly(*N*-(*N*-(2-aminoethyl)-2-aminoethyl) aspartamide) (PAsp(DET), glutathione, donor DNA, and Cas9 RNP complexes (PAsp(DET)) that increase in vitro and in vivo endosomal escape in DNA repair mechanism. This research revealed a benchmark study for the efficient nonviral delivery for HDR in treating genetic disorders (Deng et al. 2019).

12.5 Critical Challenges for Nonviral Delivery of CRISPR System

Compared with other efficient delivering approaches, the native CRISPR/Cas systems circumvent stringent obstacles for in vivo applications. The first challenge is the active integration of genetic payloads in vector delivery due to possessing large size and presence of various charge characteristics of donor DNA, mRNA, and Cas9 protein, respectively (Subburaj et al. 2016). Hence, cationic polymers and lipids are more likely prone to encapsulate negatively charged proteins that cannot be feasible to deliver Cas9 proteins. Like other types of protein, large size (~4.5 kb) of DNA and Cas9/mRNA also triggers hindrance for the potential encapsulation application. Although the novel and newly generated Cas9 system isolated from the specie *Staphylococcus aureus* strain (SaCas9) possesses 1 kb in size that is shorter than previously developed SpCas9, the average size of genetic payloads is still too large for genome editing applications (Kleinstiver et al. 2015). Furthermore, CRISPR tools integrated into nonviral vectors must be stable during the extracellular and

intracellular transport until they reach the desired target sites. A nonviral delivery platform consisting of CRISPR/Cas9 tools at first meets extracellular degradation components such as RNases, proteases, DNases in the blood, activation of cytokines, signaling of immune cells, and phagocytosis by macrophages (Li et al. 1999). Second, the effective signaling of the CRISPR/Cas9 tools to recognize the target-specific site and remove the extravasation out from the bloodstream to mitigate the off-target effects and enhance the efficiency of gene editing in the untargeted locus. Furthermore, cell-penetrating peptides or target-specific ligands can be chemically assembled on the surface of NPs and easily trafficked and reached to the membrane of the target sites to attain desired gene editing (Cabral et al. 2011). After the NPs are attached to the desired cell lines, the third obstacle rapidly escapes the NPs from the endosomal barrier (pH ~ 5.0) to circumvent the degradation from the endosomal effect (Harush-Frenkel et al. 2007). Subsequently, the system based on CRISPR/Cas9-plasmid-DNA complexes must ameliorate many challenges as compared to CRISPR/Cas9 RNP or mRNA complexes because the ability of plasmid DNA needs to cross nucleus membranes to transcribe the nucleus likewise, in the same manner actively, get entry to the cytoplasm to translate the Cas9 proteins and efficiently edit the desired target genome site (Chen et al. 2020).

12.6 Conclusion

CRISPR/Cas9 genome editing system is one of the most robust editing tools, which potentially simplifies previous gene manipulation strategies. Recently, this system has been extensively applied for vector delivery to achieve transgene-free gene-editing applications. Besides this, the modification of the vector delivery system is also one of the serious concerns. The delivery through viral vectors showed various issues of their own such as high cost and restriction in packaging efficiency. Therefore, the construction of nonviral vectors is an efficient and safe delivery approach with significant applications. The nonviral vectors showed hindrance to trigger the valuable advantages for the CRISPR/Cas9 genetic cargoes that include large-sized packaging stability, high retention time, and preventing safekeeping from the degradation through enzymes during transportation (Wan et al. 2019). Though the synthesized lipids, polymerics, and gold nanoparticles have demonstrated rapid progression for all delivery approaches of CRISPR-mediated payloads, for the targeted delivery of various gene-editing components, the applications of novel NPs substantially investigated more to achieve tunable desired translation for genome editing. Overall, despite these obstacles, the novel and rapid achievements in the field of gene editing and tailored nano-based vector delivery will significantly facilitate the shortcomings to accelerate the clinical translation of CRISPR-based transgene-free editing shortly in the near future.

Conflict of Interest The authors declared that they have no conflict of interest.

References

Ahmar S, Saeed S, Khan MHU, Ullah Khan S, Mora-Poblete F, Kamran M, Faheem A, Maqsood A, Rauf M, Saleem S, Hong WJ (2020) A revolution toward gene-editing technology and its application to crop improvement. Int J Mol Sci 21(16):5665

Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P (2007) CRISPR provides acquired resistance against viruses in prokaryotes. Science 315(5819):1709–1712

Bikard D, Euler CW, Jiang W, Nussenzweig PM, Goldberg GW, Duportet X, Fischetti VA, Marraffini LA (2014) Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. Nat Biotechnol 32(11):1146–1150

Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV, Van Der Oost J (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 321(5891):960–964

Cabral H, Matsumoto Y, Mizuno K, Chen Q, Murakami M, Kimura M, Terada Y, Kano M, Miyazono K, Uesaka MJNN, Nishiyama N (2011) Accumulation of sub-100 nm polymeric micelles in poorly permeable tumours depends on size. Nat Nanotechnol 6(12):815–823

Cardarelli F, Pozzi D, Bifone A, Marchini C, Caracciolo G (2012) Cholesterol-dependent macropinocytosis and endosomal escape control the transfection efficiency of lipoplexes in CHO living cells. Mol Pharm 9(2):334–340

Chakraborty S, Ji H, Kabadi AM, Gersbach CA, Christoforou N, Leong KW (2014) A CRISPR/Cas9-based system for reprogramming cell lineage specification. Stem Cell Rep 3(6):940–947

Chamberlain K, Riyad JM, Weber T (2016) Expressing transgenes that exceed the packaging capacity of adeno-associated virus capsids. Human Gene Ther Methods 27(1):1–12

Chen X, Gonçalves MA (2016) Engineered viruses as genome editing devices. Mol Ther 24(3):447–457

Chen F, Zhang J, Wang L, Wang Y, Chen M (2015) Tumor pH e-triggered charge-reversal and redox-responsive nanoparticles for docetaxel delivery in hepatocellular carcinoma treatment. Nanoscale 7(38):15763–15779

Chen Z, Liu F, Chen Y, Liu J, Wang X, Chen AT, Deng G, Zhang H, Liu J, Hong Z, Zhou J (2017) Targeted delivery of CRISPR/Cas9-mediated cancer gene therapy via liposome-templated hydrogel nanoparticles. Adv Funct Mater 27(46):1703036

Chen F, Alphonse MP, Liu Y, Liu Q (2019) Targeting mutant KRAS for anticancer therapy. Curr Top Med Chem 19(23):2098–2113

Chen F, Alphonse M, Liu Q (2020) Strategies for nonviral nanoparticle-based delivery of CRISPR/Cas9 therapeutics. Wiley Interdiscip Rev Nanomed Nanobiotechnol 12(3):e1609

Cho SW, Kim S, Kim JM, Kim JS (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol 31(3):230–232

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121):819–823

Deng H, Huang W, Zhang Z (2019) Nanotechnology based CRISPR/Cas9 system delivery for genome editing: progress and prospect. Nano Res 12(10):2437–2450

Dever DP, Bak RO, Reinisch A, Camarena J, Washington G, Nicolas CE, Pavel-Dinu M, Saxena N, Wilkens AB, Mantri S, Uchida N (2016) CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. Nature 539(7629):384–389

Evers MJ, Kulkarni JA, van der Meel R, Cullis PR, Vader P, Schiffelers RM (2018) State-of-the-art design and rapid-mixing production techniques of lipid nanoparticles for nucleic acid delivery. Small Method 2(9):1700375

Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M (1987) Lipofection: a highly efficient, lipidmediated DNA-transfection procedure. PNAS 84(21):7413–7417

Fineran PC, Charpentier E (2012) Memory of viral infections by CRISPR-Cas adaptive immune systems: acquisition of new information. Virology 434(2):202–209
Garneau JE, Dupuis M-È, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH, Moineau S (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 468(7320):67–71
Gasiunas G, Siksnys V (2013) RNA-dependent DNA endonuclease Cas9 of the CRISPR system: Holy Grail of genome editing? Trends Microbiol 21(11):562–567
Gasiunas G, Barrangou R, Horvath P, Siksnys V (2012) Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. PNAS 109(39):E2579–E2586
Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154(2):442–451
Givens BE, Naguib YW, Geary SM, Devor EJ, Salem AK (2018) Nanoparticle-based delivery of CRISPR/Cas9 genome-editing therapeutics. AAPS J 20(6):1–22
Guilinger JP, Thompson DB, Liu DR (2014) Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. Nat Biotechnol 32(6):577–582
Hale CR, Zhao P, Olson S, Duff MO, Graveley BR, Wells L, Terns RM, Terns MP (2009) RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. Cell 139(5):945–956
Harush-Frenkel O, Debotton N, Benita S, Altschuler Y (2007) Targeting of nanoparticles to the clathrin-mediated endocytic pathway. Biochem Biophys Res Commun 353(1):26–32
Horvath P, Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. Science 327(5962):167–170
Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157(6):1262–1278
Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JJ, Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31(3):227–229
Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821
Kang YK, Kwon K, Ryu JS, Lee HN, Park C, Chung HJ (2017) Nonviral genome editing based on a polymer-derivatized CRISPR nanocomplex for targeting bacterial pathogens and antibiotic resistance. Bioconjug Chem 28(4):957–967
Kay MA (2011) State-of-the-art gene-based therapies: the road ahead. Nat Rev Genet 12(5):316–328
Kish PE, Tsume Y, Kijek P, Lanigan TM, Hilfinger JM, Roessler BJ (2007) Bile acid-oligopeptide conjugates interact with DNA and facilitate transfection. Mol Pharm 4(1):95–103
Kleinstiver BP, Prew MS, Tsai SQ, Nguyen NT, Topkar VV, Zheng Z, Joung JK (2015) Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition. Nat Biotechnol 33(12):1293–1298
Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JKJN (2016) High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature 529(7587):490–495
Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS, Qi LS (2013) CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat Protoc 8(11):2180–2196
Lee K, Conboy M, Park HM, Jiang F, Kim HJ, Dewitt MA, Mackley VA, Chang K, Rao A, Skinner C, Shobha T (2017) Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. Nat Biomed Eng 1(11):889–901
Leung AK, Tam YYC, Cullis PR (2014) Lipid nanoparticles for short interfering RNA delivery. Adv Genet 88:71–110
Li S, Tseng WC, Stolz DB, Wu SP, Watkins SC, Huang L (1999) Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection. Gene Ther 6(4):585–594

Li L, Wei Y, Gong C (2015) Polymeric nanocarriers for non-viral gene delivery. J Biomed Nanotechnol 11(5):739–770

Li L, Hu S, Chen X (2018) Non-viral delivery systems for CRISPR/Cas9-based genome editing: challenges and opportunities. Biomaterials 171:207–218

Liu Q, Zhu H, Liu Y, Musetti S, Huang L (2018a) BRAF peptide vaccine facilitates therapy of murine BRAF-mutant melanoma. Cancer Immunol Immunother 67(2):299–310

Liu Q, Zhu H, Tiruthani K, Shen L, Chen F, Gao K, Zhang X, Hou L, Wang D, Liu R, Huang L (2018b) Nanoparticle-mediated trapping of Wnt family member 5A in tumor microenvironments enhances immunotherapy for B-Raf proto-oncogene mutant melanoma. ACS Nano 12(2):1250–1261

Liu C, Wan T, Wang H, Zhang S, Ping Y, Cheng Y (2019) A boronic acid-rich dendrimer with robust and unprecedented efficiency for cytosolic protein delivery and CRISPR-Cas9 gene editing. Sci Adv 5(6):eaaw8922

Long C, Amoasii L, Mireault AA, McAnally JR, Li H, Sanchez-Ortiz E, Bhattacharyya S, Shelton JM, Bassel-Duby R, Olson EN (2016) Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. Science 351(6271):400–403

Lotti SN (2017) Using CRISPR/Cas9 to modify the genome of cattle. University of Illinois at Urbana-Champaign, Urbana

Makarova KS, Aravind L, Wolf YI, Koonin EV (2011a) Unification of Cas protein families and a simple scenario for the origin and evolution of CRISPR-Cas systems. Biol Direct 6(1):1–27

Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, Moineau S, Mojica FJ, Wolf YI, Yakunin AF, Van Der Oost J (2011b) Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol 9(6):467–477

Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM (2013) RNA-guided human genome engineering via Cas9. Science 339(1621):823–826

Mintzer MA, Simanek EE (2009) Nonviral vectors for gene delivery. Chem Rev 109(2):259–302

Mohammadinejad R, Dehshahri A, Madamsetty VS, Zahmatkeshan M, Tavakol S, Makvandi P, Khorsandi D, Pardakhty A, Ashrafizadeh M, Afshar EG, Zarrabi A (2020) In vivo gene delivery mediated by non-viral vectors for cancer therapy. J Control Release 325:249–275

Möller K, Müller K, Engelke H, Bräuchle C, Wagner E, Bein T (2016) Highly efficient siRNA delivery from core-shell mesoporous silica nanoparticles with multifunctional polymer caps. Nanoscale 8(7):4007–4019

Mout R, Rotello VM (2017) Cytosolic and nuclear delivery of CRISPR/Cas9-ribonucleoprotein for gene editing using arginine functionalized gold nanoparticles. Bio Protoc 7(20):e2586

Mout R, Ray M, Yesilbag Tonga G, Lee YW, Tay T, Sasaki K, Rotello VM (2017) Direct cytosolic delivery of CRISPR/Cas9-ribonucleoprotein for efficient gene editing. ACS Nano 11(3):2452–2458

Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S (2013) Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nat Biotechnol 31(8):691–693

Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP (2014) Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156(4):836–843

Onuki Y, Obata Y, Kawano K, Sano H, Matsumoto R, Hayashi Y, Takayama K (2016) Membrane microdomain structures of liposomes and their contribution to the cellular uptake efficiency into HeLa cells. Mol Pharm 13(2):369–378

Pack DW, Hoffman AS, Pun S, Stayton PS (2005) Design and development of polymers for gene delivery. Nat Rev Drug Discov 4(7):581–593

Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152(5):1173–1183

Qiao J, Sun W, Lin S, Jin R, Ma L, Liu Y (2019) Cytosolic delivery of CRISPR/Cas9 ribonucleoproteins for genome editing using chitosan-coated red fluorescent protein. Chem Commun 55(32):4707–4710

Ramamoorth M, Narvekar A (2015) Non viral vectors in gene therapy-an overview. J Clin Diagn Res 9(1):GE01

Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F (2013a) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154(6):1380–1389

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013b) Genome engineering using the CRISPR-Cas9 system. Nat Protoc 8(11):2281–2308

Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV (2015) In vivo genome editing using Staphylococcus aureus Cas9. Nature 520(7546):186–191

Ryu N, Kim MA, Park D, Lee B, Kim YR, Kim KH, Baek JI, Kim WJ, Lee KY, Kim UK (2018) Effective PEI-mediated delivery of CRISPR-Cas9 complex for targeted gene therapy. Nanomedicine 14(7):2095–2102

Sapranauskas R, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V (2011) The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. Nucleic Acids Res 39(21):9275–9282

Schmidt F, Grimm D (2015) CRISPR genome engineering and viral gene delivery: a case of mutual attraction. Biotechnol J 10(2):258–272

Schumann K, Lin S, Boyer E, Simeonov DR, Subramaniam M, Gate RE, Haliburton GE, Chun JY, Bluestone JA, Doudna JA, Marson A (2015) Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. PNAS 112(33):10437–10442

Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, Wang L, Hodgkins A, Iyer V, Huang X, Skarnes WC (2014) Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nat Methods 11(4):399–402

Spoelstra WK, Jacques JM, Gonzalez-Linares R, Nobrega FL, Haagsma AC, Dogterom M, Meijer DH, Idema T, Brouns SJ, Reese L (2021) CRISPR-based DNA and RNA detection with liquid-liquid phase separation. Biophys J 120(7):1198–1209

Subburaj S, Chung SJ, Lee C, Ryu SM, Kim DH, Kim JS, Bae S, Lee GJ (2016) Site-directed mutagenesis in petunia × hybrida protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. Plant Cell Rep 35(7):1535–1544

Sun W, Ji W, Hall JM, Hu Q, Wang C, Beisel CL, Gu Z (2015) Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing. Angew Chem 127(41):12197–12201

Wahane A, Waghmode A, Kapphahn A, Dhuri K, Gupta A, Bahal R (2020) Role of lipid-based and polymer-based non-viral vectors in nucleic acid delivery for next-generation gene therapy. Molecules 25(12):2866

Wan T, Niu D, Wu C, Xu FJ, Church G, Ping Y (2019) Material solutions for delivery of CRISPR/Cas-based genome editing tools: current status and future outlook. Mater Today 26:40–66

Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153(4):910–918

Wang P, Zhang L, Zheng W, Cong L, Guo Z, Xie Y, Wang L, Tang R, Feng Q, Hamada Y, Gonda K (2018) Thermo-triggered release of CRISPR-Cas9 system by lipid-encapsulated gold nanoparticles for tumor therapy. Angew Chem Int Ed 57(6):1491–1496

Weber T, Wefers B, Wurst W, Sander S, Rajewsky K, Kühn R (2015) Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nat Biotechnol 33(5):543–548

Whitehead KA, Langer R, Anderson DG (2009) Knocking down barriers: advances in siRNA delivery. Drug Discov 8(2):129–138

Wiedenheft B, Sternberg SH, Doudna JA (2012) RNA-guided genetic silencing systems in bacteria and archaea. Nature 482(7385):331–338

Wu Y, Zhou H, Fan X, Zhang Y, Zhang M, Wang Y, Xie Z, Bai M, Yin Q, Liang D, Tang W (2015) Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. Cell Res 25(1):67–79

Xue W, Chen S, Yin H, Tammela T, Papagiannakopoulos T, Joshi NS, Cai W, Yang G, Bronson R, Crowley DG, Zhang F (2014) CRISPR-mediated direct mutation of cancer genes in the mouse liver. Nature 514(7522):380–384

Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R (2013) One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154(6):1370–1379

Yin H, Song C-Q, Dorkin JR, Zhu LJ, Li Y, Wu Q, Park A, Yang J, Suresh S, Bizhanova A, Gupta A (2016) Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. Nat Biotechnol 34(3):328–333

Yin H, Song C-Q, Suresh S, Wu Q, Walsh S, Rhym LH, Mintzer E, Bolukbasi MF, Zhu LJ, Kauffman K, Mou H (2017) Structure-guided chemical modification of guide RNA enables potent non-viral in vivo genome editing. Nat Biotechnol 35(12):1179–1187

Yu C, Liu Y, Ma T, Liu K, Xu S, Zhang Y, Liu H, La Russa M, Xie M, Ding S, Qi LS (2015) Small molecules enhance CRISPR genome editing in pluripotent stem cells. Cell Stem Cell 16(2):142–147

Yu W, Mookherjee S, Chaitankar V, Hiriyanna S, Kim JW, Brooks M, Ataeijannati Y, Sun X, Dong L, Li T, Swaroop A (2017) Nrl knockdown by AAV-delivered CRISPR/Cas9 prevents retinal degeneration in mice. Nat Commun 8(1):1–15

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van Der Oost J, Regev A, Koonin EV (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163(3):759–771

Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, DeGennaro EM, Winblad N, Choudhury SR, Abudayyeh OO, Gootenberg JS, Wu WY (2017) Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. Nat Biotechnol 35(1):31–34

Zhang Z, Wan T, Chen Y, Chen Y, Sun H, Cao T, Songyang Z, Tang G, Wu C, Ping Y, Xu FJ (2019) Cationic polymer-mediated CRISPR/Cas9 plasmid delivery for genome editing. Macromol Rapid Commun 40(5):p1800068

Zou L, Chen F, Bao J, Wang S, Wang L, Chen M, He C, Wang Y (2016) Preparation, characterization, and anticancer efficacy of evodiamine-loaded PLGA nanoparticles. Drug Deliv 23(3):898–906

Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, Maeder ML, Joung JK, Chen ZY, Liu DR (2015) Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat Biotechnol 33(1):73–80